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PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE

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PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE

This application is a continuation-in-part of co-pending U.S. Patent Application Serial No. 08/318,905 filed October 6, 1994, which in turn is a continuation-in-part of co-pending U.S. Patent Application Serial No. 08/133,803 filed October 6, 1993.

FIELD OF THE INVENTION

The present invention relates generally to platelet-activating factor acetylhydrolase and more specifically to novel purified and isolated polynucleotides encoding human plasma platelet-activating factor acetylhydrolase, to the platelet-activating factor acetylhydrolase products encoded by the polynucleotides, to materials and methods for the recombinant production of platelet-activating factor acetylhydrolase products and to antibody substances specific for platelet-activating factor acetylhydrolase.

BACKGROUND

Platelet-activating factor (PAF) is a biologically active phospholipid synthesized by various cell types. *In vivo* and at normal concentrations of 10^{-10} to 10^{-9} M, PAF activates target cells such as platelets and neutrophils by binding to specific G protein-coupled cell surface receptors [Venable *et al.*, *J. Lipid Res.*, *34*: 691-701 (1993)]. PAF has the structure 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine. For optimal biological activity, the sn-1 position of the PAF glycerol backbone must be in an ether linkage with a fatty alcohol and the sn-3 position must have a phosphocholine head group.

PAF functions in normal physiological processes (*e.g.*, inflammation, hemostasis and parturition) and is implicated in pathological inflammatory responses (*e.g.*, asthma, anaphylaxis, septic shock and arthritis) [Venable *et al.*, *supra*, and Lindsberg *et al.*, *Ann. Neurol.*, *30*: 117-129 (1991)]. The likelihood of PAF involvement in pathological responses has prompted attempts to modulate the activity of PAF and the major focus of these attempts has been the development of antagonists of PAF activity which interfere with binding of PAF to cell surface receptors. See, for example, Heuer *et al.*, *Clin. Exp. Allergy*, *22*: 980-983 (1992).

The synthesis and secretion of PAF as well as its degradation and clearance appear to be tightly controlled. To the extent that pathological inflammatory actions of PAF result from a failure of PAF regulatory mechanisms giving rise to excessive production, inappropriate production or lack of degradation, an alternative means of modulating the activity of PAF would involve mimicing or augmenting the natural process by which resolution of inflammation occurs. Macrophages [Stafforini *et al.*, *J. Biol. Chem.*, *265*(17): 9682-9687 (1990)], hepatocytes and the human hepatoma cell line HepG2 [Satoh *et al.*, *J. Clin. Invest.*, *87*: 476-481 (1991) and Tarbet *et al.*, *J. Biol. Chem.*, *266*(25): 16667-16673 (1991)] have been reported to release an enzymatic activity, PAF acetylhydrolase (PAF-AH), that inactivates PAF. In addition to inactivating PAF, PAF-AH also inactivates oxidatively fragmented phospholipids such as products of the arachidonic acid cascade that mediate inflammation. See, Stremler *et al.*, *J. Biol. Chem.*, *266*(17): 11095-11103 (1991). The inactivation of PAF by PAF-AH occurs primarily by hydrolysis of the PAF sn-2 acetyl group and PAF-AH metabolizes oxidatively fragmented phospholipids by removing sn-2 acyl groups. Two types of PAF-AH have been identified: cytoplasmic forms found in a variety of cell types and tissues such as endothelial cells and erythrocytes, and an extracellular form found in plasma and serum. Plasma PAF-AH does not hydrolyze intact phospholipids except for PAF and this substrate specificity allows the enzyme to circulate *in vivo* in a fully active state without adverse effects. The plasma PAF-AH appears to account for all of the PAF degradation in human blood *ex vivo* [Stafforini *et al.*, *J. Biol. Chem.*, *262*(9): 4223-4230 (1987)].

While the cytoplasmic and plasma forms of PAF-AH appear to have identical substrate specificity, plasma PAF-AH has biochemical characteristics which distinguish it from cytoplasmic PAF-AH and from other characterized lipases. Specifically, plasma PAF-AH is associated with lipoprotein particles, is inhibited by diisopropyl fluorophosphate, is not affected by calcium ions, is relatively insensitive to proteolysis, and has an apparent molecular weight of 43,000 daltons. See, Stafforini *et al.* (1987), *supra*. The same Stafforini *et al.* article describes a procedure for partial purification of PAF-AH from human plasma and the amino acid composition of the plasma material obtained by use of the procedure. Cytoplasmic

PAF-AH has been purified from erythrocytes as reported in Stafforini *et al.*, *J. Biol. Chem.*, *268*(6): 3857-3865 (1993) and ten amino terminal residues of cytoplasmic PAF-AH are also described in the article. Hattori *et al.*, *J. Biol. Chem.*, *268*(25): 18748-18753 (1993) describes the purification of cytoplasmic PAF-AH from bovine brain. Subsequent to filing of the parent application hereto the nucleotide sequence of bovine brain cytoplasmic PAF-AH was published in Hattori *et al.*, *J. Biol. Chem.*, *269*(237): 23150-23155 (1994). On January 5, 1995, three months after the filing date of the parent application hereto, a nucleotide sequence for a lipoprotein associated phospholipase A_2 (Lp-PLA_2) was published in Smithkline Beecham PLC Patent Cooperation Treaty (PCT) International Publication No. WO 95/00649. The nucleotide sequence of the Lp-PLA_2 differs at one position when compared to the nucleotide sequence of the PAF-AH of the present invention. The nucleotide difference (corresponding to position 1297 of SEQ ID NO: 7) results in an amino acid difference between the enzymes encoded by the polynucleotides. The amino acid at position 379 of SEQ ID NO: 8 is a valine while the amino acid at the corresponding position in Lp-PLA_2 is an alanine. In addition, the nucleotide sequence of the PAF-AH of the present invention includes 124 bases at the 5' end and twenty bases at the 3' end not present in the Lp-PLA_2 sequence. Three months later, on April 10, 1995, a Lp-PLA_2 sequence was deposited in GenBank under Accession No. U24577 which differs at eleven positions when compared to the nucleotide sequence of the PAF-AH of the present invention. The nucleotide differences (corresponding to position 79, 81, 84, 85, 86, 121, 122, 904, 905, 911, 983 and 1327 of SEQ ID NO: 7) results in four amino acid differences between the enzymes encoded by the polynucleotides. The amino acids at positions 249, 250, 274 and 389 of SEQ ID NO: 8 are lysine, aspartic acid, phenylalanine and leucine, respectively, while the respective amino acid at the corresponding positions in the GenBank sequence are isoleucine, arginine, leucine and serine.

The recombinant production of PAF-AH would make possible the use of exogenous PAF-AH to mimic or augment normal processes of resolution of inflammation *in vivo*. The administration of PAF-AH would provide a physiological advantage over administration of PAF receptor antagonists because PAF-AH is a product normally found in plasma. Moreover, because PAF receptor antagonists

which are structurally related to PAF inhibit native PAF-AH activity, the desirable metabolism of PAF and of oxidatively fragmented phospholipids is thereby prevented. Thus, the inhibition of PAF-AH activity by PAF receptor antagonists counteracts the competitive blockade of the PAF receptor by the antagonists. See, Stremler *et al.*, *supra*. In addition, in locations of acute inflammation, for example, the release of oxidants results in inactivation of the native PAF-AH enzyme in turn resulting in elevated local levels of PAF and PAF-like compounds which would compete with any exogenously administed PAF receptor antagonist for binding to the PAF receptor. In contrast, treatment with recombinant PAF-AH would augment endogenous PAF-AH activity and compensate for any inactivated endogenous enzyme.

There thus exists a need in the art to identify and isolate polynucleotide sequences encoding human plasma PAF-AH, to develop materials and methods useful for the recombinant production of PAF-AH and to generate reagents for the detection of PAF-AH in plasma.

SUMMARY OF THE INVENTION

The present invention provides novel purified and isolated polynucleotides (*i.e.*, DNA and RNA both sense and antisense strands) encoding human plasma PAF-AH or enzymatically active fragments thereof. Preferred DNA sequences of the invention include genomic and cDNA sequences as well as wholly or partially chemically synthesized DNA sequences. The DNA sequence encoding PAF-AH that is set out in SEQ ID NO: 7 and DNA sequences which hybridize to the noncoding strand thereof under standard stringent conditions or which would hybridize but for the redundancy of the genetic code, are contemplated by the invention. Also contemplated by the invention are biological replicas (i.e., copies of isolated DNA sequences made *in vivo* or *in vitro*) of DNA sequences of the invention. Autonomously replicating recombinant constructions such as plasmid and viral DNA vectors incorporating PAF-AH sequences and especially vectors wherein DNA encoding PAF-AH is operatively linked to an endogenous or exogenous expression control DNA sequence and a transcription terminator are also provided.

According to another aspect of the invention, procaryotic or eucaryotic host cells are stably transformed with DNA sequences of the invention in a manner

allowing the desired PAF-AH to be expressed therein. Host cells expressing PAF-AH products can serve a variety of useful purposes. Such cells constitute a valuable source of immunogen for the development of antibody substances specifically immunoreactive with PAF-AH. Host cells of the invention are conspicuously useful in methods for the large scale production of PAF-AH wherein the cells are grown in a suitable culture medium and the desired polypeptide products are isolated from the cells or from the medium in which the cells are grown by, for example, immunoaffinity purification.

A non-immunological method contemplated by the invention for purifying PAF-AH from plasma includes the following steps: (a) isolating low density lipoprotein particles; (b) solubilizing said low density lipoprotein particles in a buffer comprising 10mM CHAPS to generate a first PAF-AH enzyme solution; (c) applying said first PAF-AH enzyme solution to a DEAE anion exchange column; (d) washing said DEAE anion exchange column using an approximately pH 7.5 buffer comprising 1mM CHAPS; (e) eluting PAF-AH enzyme from said DEAE anion exchange column in fractions using approximately pH 7.5 buffers comprising a gradient of 0 to 0.5 M NaCl; (f) pooling fractions eluted from said DEAE anion exchange column having PAF-AH enzymatic activity; (g) adjusting said pooled, active fractions from said DEAE anion exchange column to 10mM CHAPS to generate a second PAF-AH enzyme solution; (h) applying said second PAF-AH enzyme solution to a blue dye ligand affinity column; (i) eluting PAF-AH enzyme from said blue dye ligand affinity column using a buffer comprising 10mM CHAPS and a chaotropic salt; (j) applying the eluate from said blue dye ligand affinity column to a Cu ligand affinity column; (k) eluting PAF-AH enzyme from said Cu ligand affinity column using a buffer comprising 10mM CHAPS and imidazole; (l) subjecting the eluate from said Cu ligand affinity column to SDS-PAGE; and (m) isolating the approximately 44 kDa PAF-AH enzyme from the SDS-polyacrylamide gel. Preferably, the buffer of step (b) is 25 mM Tris-HCl, 10mM CHAPS, pH 7.5; the buffer of step (d) is 25 mM Tris-HCl, 1mM CHAPS; the column of step (h) is a Blue Sepharose Fast Flow column; the buffer of step (i) is 25mM Tris-HCl, 10mM CHAPS, 0.5M KSCN, pH 7.5; the column of step (j) is a Cu Chelating Sepharose

column; and the buffer of step (k) is 25 mM Tris-HCl, 10mM CHAPS, 0.5M NaCl, 50mM imidazole at a pH in a range of about pH 7.5-8.0.

A method contemplated by the invention for purifying enzymatically-active PAF-AH from *E. coli* producing PAF-AH includes the steps of: (a) preparing a centrifugation supernatant from lysed *E. coli* producing PAF-AH enzyme; (b) applying said centrifugation supernatant to a blue dye ligand affinity column; (c) eluting PAF-AH enzyme from said blue dye ligand affinity column using a buffer comprising 10mM CHAPS and a chaotropic salt; (d) applying said eluate from said blue dye ligand affinity column to a Cu ligand affinity column; and (e) eluting PAF-AH enzyme from said Cu ligand affinity column using a buffer comprising 10mM CHAPS and imidazole. Preferably, the column of step (b) is a Blue Sepharose Fast Flow column; the buffer of step (c) is 25mM Tris-HCl, 10mM CHAPS, 0.5M KSCN, pH. 7.5; the column of step (d) is a Cu Chelating Sepharose column; and the buffer of step (e) is 25mM Tris-HCl, 10mM CHAPS, 0.5M NaCl, 100mM imidazole, pH 7.5.

Another method contemplated by the invention for purifying enzymatically-active PAF-AH from *E. coli* producing PAF-AH includes the steps of: (a) preparing a centrifugation supernatant from lysed *E. coli* producing PAF-AH enzyme; (b) diluting said centrifugation supernatant in a low pH buffer comprising 10mM CHAPS; (c) applying said diluted centrifugation supernatant to a cation exchange column equilibrated at about pH 7.5; (d) eluting PAF-AH enzyme from said cation exchange column using 1M salt; (e) raising the pH of said eluate from said cation exhange column and adjusting the salt concentration of said eluate to about 0.5M salt; (f) applying said adjusted eluate from said cation exchange column to a blue dye ligand affinity column; (g) eluting PAF-AH enzyme from said blue dye ligand affinity column using a buffer comprising about 2M to about 3M salt; and (h) dialyzing said eluate from said blue dye ligand affinity column using a buffer comprising about 0.1% Tween. Preferably, the buffer of step (b) is 25mM MES, 10mM CHAPS, 1mM EDTA, pH 4.9; the column of step (c) is an S sepharose column equilibrated in 25mM MES, 10mM CHAPS, 1mM EDTA, 50mM NaCl, pH 5.5; PAF-AH is eluted in step (d) using 1mM NaCl; the pH of the eluate in step (e) is adjusted to pH 7.5 using 2M Tris base; the column in step (f) is a sepharose

column; the buffer in step (g) is 25mM Tris, 10mM CHAPS, 3M NaCl, 1mM EDTA, pH 7.5; and the buffer in step (h) is 25mM Tris, 0.5M NaCl, 0.1% Tween 80, pH 7.5.

Still another method contemplated by the invention for purifying enzymatically-active PAF-AH from *E.coli* includes the steps of: (a) preparing an *E.coli* extract which yields solubilized PAF-AH supernatant after lysis in a buffer containing CHAPS; (b) dilution of the said supernatant and application to a anion exchange column equilibrated at about pH 8.0; (c) eluting PAF-AH enzyme from said anion exchange column; (d) applying said adjusted eluate from said anion exchange column to a blue dye ligand affinity column; (e) eluting the said blue dye ligand affinity column using a buffer comprising 3.0M salt; (f) dilution of the blue dye eluate into a suitable buffer for performing hydroxylapatite chromatography; (g) performing hydroxylapatite chromatography where washing and elution is accomplished using buffers (with or without CHAPS); (h) diluting said hydroxylapatite eluate to an appropriate salt concentration for cation exchange chromatography; (i) applying said diluted hydroxylapatite eluate to a cation exchange column at a pH ranging between approximately 6.0 to 7.0; (j) elution of PAF-AH from said cation exchange column with a suitable formulation buffer; (k) performing cation exchange chromatography in the cold; and (l) formulation of PAF-AH in liquid or frozen form in the absence of CHAPS.

Preferably in step (a) above the lysis buffer is 25mM Tris, 100mM NaCl, 1mM EDTA, 20mM CHAPS, pH 8.0; in step (b) the dilution of the supernatant for anion exchange chromatography is 3-4 fold into 25mM Tris, 1mM EDTA, 10mM CHAPS, pH 8.0 and the column is a Q-Sepharose column equilibrated with 25mM Tris, 1mM EDTA, 50mM NaCl, 10mM CHAPS, pH 8.0; in step (c) the anion exchange column is eluted using 25mM Tris, 1mM EDTA, 350mM NaCl, 10mM CHAPS, pH 8.0; in step (d) the eluate from step (c) is applied directly onto a blue dye affinity column; in step (e) the column is eluted with 3M NaCl, 10mM CHAPS, 25mM Tris, pH 8.0 buffer; in step (f) dilution of the blue dye eluate for hydroxylapatite chromatography is accomplished by dilution into 10mM sodium phosphate, 100mM NaCl, 10mM CHAPS, pH 6.2; in step (g) hydroxylapatite chromatography is accomplished using a hydroxylapatite column equilibrated with

10mM sodium phosphate, 100mM NaCl, 10mM CHAPS and elution is accomplished using 50mM sodium phosphate, 100mM NaCl (with or without) 10mM CHAPS, pH 7.5; in step (h) dilution of said hydroxylapatite eluate for cation exchange chromatography is accomplished by dilution into a buffer ranging in pH from approximately 6.0 to 7.0 comprising sodium phosphate (with or without CHAPS); in step (i) a S Sepharose column is equilibrated with 50mM sodium phosphate, (with or without) 10mM CHAPS, pH 6.8; in step (j) elution is accomplished with a suitable formulation buffer such as potassium phosphate 50mM, 12.5mM aspartic acid, 125mM NaCl, pH 7.5 containing 0.01% Tween-80; and in step (k) cation exchange chromatrography is accomplished at 2-8°C. Examples of suitable formulation buffers for use in step (l) which stabilize PAF-AH include 50mM potassium phosphate, 12.5mM Aspartic acid, 125mM NaCl pH 7.4 (approximately, with and without the addition of Tween-80 and or Pluronic F68) or 25mM potassium phosphate buffer containing (at least) 125mM NaCl, 25mM arginine and 0.01% Tween-80 (with or without Pluronic F68 at approximately 0.1 and 0.5%).

PAF-AH products may be obtained as isolates from natural cell sources or may be chemically synthesized, but are preferably produced by recombinant procedures involving procaryotic or eucaryotic host cells of the invention. PAF-AH products having part or all of the amino acid sequence set out in SEQ ID NO: 8 are contemplated. The use of mammalian host cells is expected to provide for such post-translational modifications (*e.g.*, myristolation, glycosylation, truncation, lipidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention. PAF-AH products of the invention may be full length polypeptides, fragments or variants. Variants may comprise PAF-AH analogs wherein one or more of the specified (i.e., naturally encoded) amino acids is deleted or replaced or wherein one or more nonspecified amino acids are added: (1) without loss of one or more of the enzymatic activities or immunological characteristics specific to PAF-AH; or (2) with specific disablement of a particular biological activity of PAF-AH. Proteins or other molecules that bind to PAF-AH may be used to modulate its activity.

Also comprehended by the present invention are antibody substances (e.g., monoclonal and polyclonal antibodies, single chain antibodies, chimeric

antibodies, CDR-grafted antibodies and the like) and other binding proteins specific for PAF-AH. Specifically illustrating binding proteins of the invention are the monoclonal antibodies produced by hybridomas 90G11D and 90F2D which were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 on September 30, 1994 and were respectively assigned Accession Nos. HB 11724 and HB 11725. Also illustrating binding proteins of the invention is the monoclonal antibody produced by hybridoma 143A which was deposited with the ATCC on June 1, 1995 and assigned Accession No. HB 11900. Proteins or other molecules (*e.g.*, lipids or small molecules) which specifically bind to PAF-AH can be identified using PAF-AH isolated from plasma, recombinant PAF-AH, PAF-AH variants or cells expressing such products. Binding proteins are useful, in turn, in compositions for immunization as well as for purifying PAF-AH, and are useful for detection or quantification of PAF-AH in fluid and tissue samples by known immunological procedures. Anti-idiotypic antibodies specific for PAF-AH-specific antibody substances are also contemplated.

The scientific value of the information contributed through the disclosures of DNA and amino acid sequences of the present invention is manifest. As one series of examples, knowledge of the sequence of a cDNA for PAF-AH makes possible the isolation by DNA/DNA hybridization of genomic DNA sequences encoding PAF-AH and specifying PAF-AH expression control regulatory sequences such as promoters, operators and the like. DNA/DNA hybridization procedures carried out with DNA sequences of the invention under conditions of stringency standard in the art are likewise expected to allow the isolation of DNAs encoding allelic variants of PAF-AH, other structurally related proteins sharing one or more of the biochemical and/or immunological properties of PAF-AH, and non-human species proteins homologous to PAF-AH. The DNA sequence information provided by the present invention also makes possible the development, by homologous recombination or "knockout" strategies [see, *e.g.*, Kapecchi, *Science*, *244*: 1288-1292 (1989)], of rodents that fail to express a functional PAF-AH enzyme or that express a variant PAF-AH enzyme. Polynucleotides of the invention when suitably labelled are useful in hybridization assays to detect the capacity of cells to synthesize PAF-AH. Polynucleotides of the invention may also be the basis for diagnostic methods

useful for identifying a genetic alteration(s) in the PAF-AH locus that underlies a disease state or states. Also made available by the invention are anti-sense polynucleotides relevant to regulating expression of PAF-AH by those cells which ordinarily express the same.

Administration of PAF-AH preparations of the invention to mammalian subjects, especially humans, for the purpose of ameliorating pathological inflammatory conditions is contemplated. Based on implication of the involvement of PAF in pathological inflammatory conditions, the administration of PAF-AH is indicated, for example, in treatment of asthma [Miwa *et al.*, *J. Clin. Invest.*, *82*: 1983-1991 (1988); Hsieh *et al.*, *J. Allergy Clin. Immunol.*, *91*: 650-657 (1993); and Yamashita *et al.*, *Allergy*, *49*: 60-63 (1994)], anaphylaxis [Venable *et al.*, *supra*], shock [Venable *et al.*, *supra*], reperfusion injury and central nervous system ischemia [Lindsberg *et al.* (1991), *supra*], antigen-induced arthritis [Zarco *et al.*, *Clin. Exp. Immunol.*, *88*: 318-323 (1992)], atherogenesis [Handley *et al.*, *Drug Dev. Res.*, *7*: 361-375 (1986)], Crohn's disease [Denizot *et al.*, *Digestive Diseases and Sciences*, *37*(3): 432-437 (1992)], ischemic bowel necrosis/necrotizing enterocolitis [Denizot *et al.*, *supra* and Caplan *et al.*, *Acta Paediatr., Suppl. 396:* 11-17 (1994)], ulcerative colitis (Denizot *et al.*, *supra*), ischemic stroke [Satoh *et al.*, *Stroke*, *23*: 1090-1092 (1992)], ischemic brain injury [Lindsberg *et al.*, *Stroke*, *21*: 1452-1457 (1990) and Lindsberg *et al.* (1991), *supra*], systemic lupus erythematosus [Matsuzaki *et al.*, *Clinica Chimica Acta*, *210*: 139-144 (1992)], acute pancreatitis [Kald *et al.*, *Pancreas*, *8*(4): 440-442 (1993)], septicemia (Kald *et al.*, *supra*), acute post streptococcal glomerulonephritis [Mezzano *et al.*, *J. Am. Soc. Nephrol.*, *4*: 235-242 (1993)], pulmonary edema resulting from IL-2 therapy [Rabinovici *et al.*, *J. Clin. Invest.*, *89*: 1669-1673 (1992)], allergic inflammation [Watanabe *et al.*, *Br. J. Pharmacol.*, *111*: 123-130 (1994)], ischemic renal failure [Grino *et al.*, *Annals of Internal Medicine*, *121*(5): 345-347 (1994); preterm labor [Hoffman *et al.*, *Am. J. Obstet. Gynecol.*, *162*(2): 525-528 (1990) and Maki *et al.*, *Proc. Natl. Acad. Sci. USA*, *85*: 728-732 (1988)]; and adult respiratory distress syndrome [Rabinovici *et al.*, *J. Appl. Physiol.*, *74*(4): 1791-1802 (1993); Matsumoto *et al.*, *Clin. Exp. Pharmacol. Physiol.*, *19* 509-515 (1992); and Rodriguez-Roisin *et al.*, *J. Clin. Invest.*, *93*: 188-194 (1994)].

Animal models for many of the foregoing pathological conditions have been described in the art. For example, a mouse model for asthma and rhinitis is described in Example 16 herein; a rabbit model for arthritis is described in Zarco *et al.*, *supra*; rat models for ischemic bowel necrosis/necrotizing enterocolitis are described in Furukawa *et al.*, *Ped. Res.*, *34*,(2): 237-241 (1993) and Caplan et al., supra; a rabbit model for stroke is described in Lindsberg *et al.*, (1990), *supra*; a mouse model for lupus is described in Matsuzaki *et al.*, *supra*; a rat model for acute pancreatitis is described in Kald *et al.*, *supra*; a rat model for pulmonary edema resulting from IL-2 therapy is described in Rabinovici *et al.*, *supra*; a rat model of allergic inflammation is described in Watanabe *et al.*, *supra*); a canine model of renal allograft is described in Watson *et al.*, *Transplantation*, *56*(4): 1047-1049 (1993); and rat and guinea pig models of adult respiratory distress syndrome are respectively described in Rabinovici *et al.*, *supra*. and Lellouch-Tubiana, Am. Rev. Respir. Dis., 137: 948-954 (1988).

Specifically contemplated by the invention are PAF-AH compositions for use in methods for treating a mammal susceptible to or suffering from PAF-mediated pathological conditions comprising administering PAF-AH to the mammal in an amount sufficient to supplement endogenous PAF-AH activity and to inactivate pathological amounts of PAF in the mammal.

Therapeutic/pharmaceutical compositions contemplated by the invention include PAF-AH and a physiologically acceptable diluent or carrier and may also include other agents having anti-inflammatory effects. Dosage amounts indicated would be sufficient to supplement endogenous PAF-AH activity and to inactivate pathological amounts of PAF. For general dosage considerations see *Remmington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Co., Easton, PA (1990). Dosages will vary between about 0.1 to about 1000 μg PAF-AH/kg body weight. Therapeutic compositions of the invention may be administered by various routes depending on the pathological condition to be treated. For example, administration may be by intraveneous, subcutaneous, oral, suppository, and/or pulmonary routes.

For pathological conditions of the lung, administration of PAF-AH by the pulmonary route is particularly indicated. Contemplated for use in pulmonary administration are a wide range of delivery devices including, for example, nebulizers, metered dose inhalers, and powder inhalers, which are standard in the art.

Delivery of various proteins to the lungs and circulatory system by inhalation of aerosol formulations has been described in Adjei *et al.*, *Pharm. Res.*, *7*(6): 565-569 (1990) (leuprolide acetate); Braquet *et al.*, *J. Cardio. Pharm.*, *13*(Supp. 5): s. 143-146 (1989) (endothelin-1); Hubbard *et al.*, *Annals of Internal Medicine*, *III*(3), 206-212 (1989) (α1-antitrypsin); Smith *et al.*, *J. Clin. Invest.*, *84*: 1145-1146 (1989) (α-1-proteinase inhibitor); Debs *et al.*, *J. Immunol.*, *140*: 3482-3488 (1933) (recombinant gamma interferon and tumor necrosis factor alpha); Patent Cooperation Treaty (PCT) International Publication No. WO 94/20069 published September 15, 1994 (recombinant pegylated granulocyte colony stimulating factor).

BRIEF DESCRIPTION OF THE DRAWING

Numerous other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description thereof, reference being made to the drawing wherein:

FIGURE 1 is a photograph of a PVDF membrane containing PAF-AH purified from human plasma;

FIGURE 2 is a graph showing the enzymatic activity of recombinant human plasma PAF-AH;

FIGURE 3 is a schematic drawing depicting recombinant PAF-AH fragments and their catalytic activity;

FIGURE 4 is a bar graph illustrating blockage of PAF-induced rat foot edema by locally administered recombinant PAF-AH of the invention;

FIGURE 5 is a bar graph illustrating blockage of PAF-induced rat foot edema by intravenously administered PAF-AH;

FIGURE 6 is a bar graph showing that PAF-AH blocks PAF-induced edema but not zymosan A-induced edema;

FIGURES 7A and 7B present dose response results of PAF-AH anti-inflammatory activity in rat food edema;

FIGURES 8A and 8B present results indicating the *in vivo* efficacy of a single dose of PAF-AH over time;

FIGURE 9 is a line graph representing the pharmacokinetics of PAF-AH in rat circulation; and

FIGURE 10 is a bar graph showing the anti-inflammatory effects of PAF-AH in comparison to the lesser effects of PAF antagonists in rat foot edema.

DETAILED DESCRIPTION

The following examples illustrate the invention. Example 1 presents a novel method for the purification of PAF-AH from human plasma. Example 2 describes amino acid microsequencing of the purified human plasma PAF-AH. The cloning of a full length cDNA encoding human plasma PAF-AH is described in Example 3. Identification of a putative splice variant of the human plasma PAF-AH gene is described in Example 4. The cloning of genomic sequences encoding human plasma PAF-AH is described in Example 5. Example 6 desribes the cloning of canine, murine, bovine, chicken, rodent and macaque cDNAs homologous to the human plasma PAF-AH cDNA. Example 7 presents the results of an assay evidencing the enzymatic activity of recombinant PAF-AH transiently expressed in COS 7 cells. Example 8 describes the expression of human PAF-AH in *E. coli*, *S. cerevisiae* and mammalian cells. Example 9 presents protocols for purification of recombinant PAF-AH from *E. coli* and assays confirming its enzymatic activity. Example 10 describes various recombinant PAF-AH products including amino acid substitution analogs and amino and carboxy-truncated products, and describes experiments demonstrating that native PAF-AH isolated from plasma is glycosylated. Results of a Northern blot assay for expression of human plasma PAF-AH RNA in various tissues and cell lines are presented in Example 11 while results of *in situ* hybridzation are presented in Example 12. Example 13 describes the development of monoclonal and polyclonal antibodies specific for human plasma PAF-AH. Examples 14, 15, 16, 17 and 18 respectively describe the *in vivo* therapeutic effect of administration of recombinant PAF-AH products of the invention on acute inflammation, pleurisy, asthma, necrotizing enterocolitis, and adult respiratory distress syndrome in animal models. Example 19 presents the results of immunoassays of serum of human patients exhibiting a deficiency in PAF-AH activity and describes the identification of a genetic lesion in the patients which is apparently responsible for the deficiency.

Example 1

PAF-AH was purified from human plasma in order to provide material for amino acid sequencing.

A. Optimization of Purification Conditions

Initially, low density lipoprotein (LDL) particles were precipitated from plasma with phosphotungstate and solubilized in 0.1% Tween 20 and subjected to chromatography on a DEAE column (Pharmacia, Uppsala, Sweden) according to the method of Stafforini *et al.* (1987), *supra*, but inconsistent elution of PAF-AH activity from the DEAE column required reevaluation of the solubilization and subsequent purification conditions.

Tween 20, CHAPS (Pierce Chemical Co., Rockford, IL) and octyl glucoside were evaluated by centrifugation and gel filtration chromatography for their ability to solubilize LDL particles. CHAPS provided 25% greater recovery of solubilized activity than Tween 20 and 300% greater recovery than octyl glucoside. LDL precipitate solubilized with 10mM CHAPS was then fractionated on a DEAE Sepharose Fast Flow column (an anion exchange column; Pharmacia) with buffer containing 1mM CHAPS to provide a large pool of partially purified PAF-AH ("the DEAE pool") for evaluation of additional columns.

The DEAE pool was used as starting material to test a variety of chromatography columns for utility in further purifying the PAF-AH activity. The columns tested included: Blue Sepharose Fast Flow (Pharmacia), a dye ligand affinity column; S-Sepharose Fast Flow (Pharmacia), a cation exchange column; Cu Chelating Sepharose (Pharmacia), a metal ligand affinity column; Fractogel S (EM Separations, Gibbstown, NJ), a cation exchange column; and Sephacryl-200 (Pharmacia), a gel filtration column. These chromatographic procedures all yielded low, unsatisfactory levels of purification when operated in 1mM CHAPS. Subsequent gel filtration chromatography on Sephacryl S-200 in 1mM CHAPS generated an enzymatically active fraction which eluted over a broad size range rather than the expected 44 kDa approximate size. Taken together, these results indicated that the LDL proteins were aggregating in solution.

Different LDL samples were therefore evaluated by analytical gel filtration chromatography for aggregation of the PAF-AH activity. Samples from the

DEAE pool and of freshly solubilized LDL precipitate were analyzed on Superose 12 (Pharmacia) equilibrated in buffer with 1mM CHAPS. Both samples eluted over a very broad range of molecular weights with most of the activity eluting above 150 kDa. When the samples were then analyzed on Superose 12 equilibrated with 10mM CHAPS, the bulk of the activity eluted near 44 kDa as expected for PAF-AH activity. However, the samples contained some PAF-AH activity in the high molecular weight region corresponding to aggregates.

Other samples eluted PAF-AH activity exclusively in the approximately 44 kDa range when they were subsequently tested by gel filtration. These samples were an LDL precipitate solubilized in 10mM CHAPS in the presence of 0.5M NaCl and a fresh DEAE pool that was adjusted to 10mM CHAPS after elution from the DEAE column. These data indicate that at least 10mM CHAPS is required to maintain non-aggregated PAF-AH. Increase of the CHAPS concentration from 1mM to 10mM after chromatography on DEAE but prior to subsequent chromatographic steps resulted in dramatic differences in purification. For example, the degree of PAF-AH purification on S-Sepharose Fast Flow was increased from 2-fold to 10-fold. PAF-AH activity bound the Blue Sepharose Fast Flow column irreversibly in 1mM CHAPS, but the column provided the highest level of purification in 10mM CHAPS. The DEAE chromatography was not improved with prior addition of 10mM CHAPS.

Chromatography on Cu Chelating Sepharose after the Blue Sepharose Fast Flow column concentrated PAF-AH activity 15-fold. It was also determined that PAF-AH activity could be recovered from a reduced SDS-polyacrylamide gel, as long as samples were not boiled. The activity of material eluted from the Cu Chelating Sepharose column when subjected to SDS-polyacrylamide gel electrophoresis coincided with a major protein band when the gel was silver stained.

B. PAF-AH Purification Protocol

The novel protocol utilized to purify PAF-AH for amino acid sequencing therefore comprised the following steps which were performed at 4°C. Human plasma was divided into 900 ml aliquots in 1 liter Nalgene bottles and adjusted to pH 8.6. LDL particles were then precipitated by adding 90 ml of 3.85% sodium phosphotungstate followed by 23 ml of 2M $MgCl_2$. The plasma was then centrifuged for 15 minutes at 3600 g. Pellets were resuspended in 800 ml of 0.2%

sodium citrate. LDL was precipitated again by adding 10 g NaCl and 24 ml of 2M $MgCl_2$. LDL particles were pelleted by centrifugation for 15 minutes at 3600 g. This wash was repeated twice. Pellets were then frozen at -20°C. LDL particles from 5L of plasma were resuspended in 5 L of buffer A (25mM Tris-HCl, 10mM CHAPS, pH 7.5) and stirred overnight. Solubilized LDL particles were centrifuged at 3600 g for 1.5 hours. Supernatants were combined and filtered with Whatman 113 filter paper to remove any remaining solids. Solubilized LDL supernatant was loaded on a DEAE Sepharose Fast Flow column (11 cm x 10 cm; 1 L resin volume; 80 ml/minute) equilibrated in buffer B (25mM Tris-HCl, 1mM CHAPS, pH 7.5). The column was washed with buffer B until absorbance returned to baseline. Protein was eluted with an 8 L, 0 - 0.5M NaCl gradient and 480 ml fractions were collected. This step was necessary to obtain binding to the Blue Sepharose Fast Flow column below. Fractions were assayed for acetylhydrolase activity essentially by the method described in Example 4.

Active fractions were pooled and sufficient CHAPS was added to make the pool about 10mM CHAPS. The DEAE pool was loaded overnight at 4 ml/minute onto a Blue Sepharose Fast Flow column (5 cm x 10 cm; 200 ml bed volume) equilibrated in buffer A containing 0.5M NaCl. The column was washed with the equilibration buffer at 16 ml/minute until absorbance returned to baseline. PAF-AH activity was step eluted with buffer A containing 0.5M KSCN (a chaotropic salt) at 16 ml/minute and collected in 50 ml fractions. This step resulted in greater than 1000-fold purification. Active fractions were pooled, and the pool was adjusted to pH 8.0 with 1M Tris-HCl pH 8.0. The active pool from Blue Sepharose Fast Flow chromatography was loaded onto a Cu Chelating Sepharose column (2.5 cm x 2 cm; 10 ml bed volume; 4 ml/minute) equilibrated in buffer C [25mM Tris-HCl, 10mM CHAPS, 0.5M NaCl, pH 8.0 (pH 7.5 also worked)], and the column was washed with 50 ml buffer C. PAF-AH activity was eluted with 100 ml 50mM imidazole in buffer C and collected in 10 ml fractions. Fractions containing PAF-AH activity were pooled and dialyzed against buffer A. In addition to providing a 15-fold concentration of PAF-AH activity, the Cu Chelating Sepharose column gave a small purification. The Cu Chelating Sepharose pool was reduced in 50 mM DTT for 15 minutes at 37°C and loaded onto a 0.75 mm, 7.5% polyacrylamide gel. Gel slices

were cut every 0.5 cm and placed in disposable microfuge tubes containing 200 μl 25mM Tris-HCl, 10mM CHAPS, 150mM NaCl. Slices were ground up and allowed to incubate overnight at 4°C. The supernatant of each gel slice was then assayed for PAF-AH activity to determine which protein band on SDS-PAGE contained PAF-AH activity. PAF-AH activity was found in an approximately 44 kDa band. Protein from a duplicate gel was electrotransferred to a PVDF membrane (Immobilon-P, Millipore) and stained with Coomassie Blue. A photograph of the PVDF membrane is presented in FIGURE 1.

As presented in Table I below, approximately 200 μg PAF-AH was purified 2 x 10^6-fold from 5 L human plasma. In comparison, a 3 x 10^4-fold purification of PAF-AH activity is described in Stafforini *et al.* (1987), *supra*.

Table 1

Sample	Vol. (ml)	Activity (cpm x 10^6)	Total Activity (cpm x 10^9)	Prot. Conc. (mg/ml)	Specific Activity (cpm x 10^6)	% Recovery of Activity Step	% Recovery of Activity Cum.	Fold Purification Step	Fold Purification Cum.
Plasma	5000	23	116	62	0.37	100	100	1	1
LDL	4500	22	97	1.76	12	84	84	33	33
DEAE	4200	49	207	1.08	46	212	178	3.7	124
Blue	165	881	14	0.02	54200	70	126	1190	1.5×10^5
Cu	12	12700	152	0.15	82200	104	131	1.5	2.2×10^5
SDS-PAGE	—	—	—	—	—	—	—	~10	2.2×10^6

In summary, the following steps were unique and critical for successful purification of plasma PAF-AH for microsequencing: (1) solubilization and chromotography in 10mM CHAPS, (2) chromatography on a blue ligand affinity column such as Blue Sepharose Fast Flow, (3) chromatography on a Cu ligand affinity column such as Cu Chelating Sepharose, and (4) elution of PAF-AH from SDS-PAGE.

Example 2

For amino acid sequencing, the approximately 44 kDa protein band from the PAF-AH- containing PVDF membrane described in Example 1 was excised and sequenced using an Applied Biosystems 473A Protein sequencer. N-terminal sequence analysis of the approximately 44 kDa protein band corresponding to the PAF-AH activity indicated that the band contained two major sequences and two minor sequences. The ratio of the two major sequences was 1:1 and it was therefore difficult to interpret the sequence data.

To distinguish the sequences of the two major proteins which had been resolved on the SDS gel, a duplicate PVDF membrane containing the approximately 44 kDa band was cut in half such that the upper part and the lower part of the membrane were separately subjected to sequencing.

The N-terminal sequence obtained for the lower half of the membrane was:

SEQ ID NO: 1

F K D L G E E N F K A L V L I A F

A search of protein databases revealed this sequence to be a fragment of human serum albumin. The upper half of the same PVDF membrane was also sequenced and the N-terminal amino acid sequence determined was:

SEQ ID NO: 2

I Q V L M A A A S F G Q T K I P

This sequence did not match any protein in the databases searched and was different from the N-terminal amino acid sequence:

SEQ ID NO: 3

M K P L V V F V L G G

which was reported for erythrocyte cytoplasmic PAF-AH in Stafforini *et al.* (1993), *supra*. The novel sequence (SEQ ID NO: 2) was utilized for cDNA cloning of human plasma PAF-AH as described below in Example 3.

Example 3

A full length clone encoding human plasma PAF-AH was isolated from a macrophage cDNA library.

A. Construction of a Macrophage cDNA Library

Poly A$^+$ RNA was harvested from peripheral blood monocyte-derived macrophages. Double-stranded, blunt-ended cDNA was generated using the Invitrogen Copy Kit (San Diego, CA) and *Bst*XI adapters were ligated to the cDNA prior to insertion into the mammalian expression vector, pRc/CMV (Invitrogen). The resulting plasmids were introduced into *E. coli* strain XL-1 Blue by electroporation. Transformed bacteria were plated at a density of approximately 3000 colonies per agarose plate on a total of 978 plates. Plasmid DNA prepared separately from each plate was retained in individual pools and was also combined into larger pools representing 300,000 clones each.

B. Library Screening by PCR

The macrophage library was screened by the polymerase chain reaction utilizing a degenerate antisense oligonucleotide PCR primer based on the novel N-terminal amino acid sequence described in Example 2. The sequence of the primer is set out below in IUPAC nomenclature and where "I" is an inosine.

SEQ ID NO: 4

5' ACATGAATTCGGIATCYTTIGTYTGICCRAA 3'

The codon choice tables of Wada *et al.*, *Nuc. Acids Res.*, *19S*: 1981-1986 (1991) were used to select nucleotides at the third position of each codon of the primer. The primer was used in combination with a primer specific for either the SP6 or T7 promoter sequences, both of which flank the cloning site of pRc/CMV, to screen the macrophage library pools of 300,000 clones. All PCR reactions contained 100 ng of template cDNA, 1 μg of each primer, 0.125mM of each dNTP, 10mM Tris-HCl pH 8.4, 50mM $MgCl_2$ and 2.5 units of Taq polymerase. An initial denaturation step of 94°C for four minutes was followed by 30 cycles of amplification of 1 minute at 94°C, 1 minute at 60°C and 2 minutes at 72°C. The resulting PCR product was cloned into pBluescript SK$^-$ (Stratagene, La Jolla, CA) and its nucleotide sequence determined by the dideoxy chain termination method. The PCR product contained the sequence predicted by the novel peptide sequence and corresponds to nucleotides 1 to 331 of SEQ ID NO: 7.

The PCR primers set out below, which are specific for the cloned PCR fragment described above, were then designed for identifying a full length clone.

Sense Primer (SEQ ID NO: 5)

5' TATTTCTAGAAGTGTGGTGGAACTCGCTGG 3'

Antisense Primer (SEQ ID NO: 6)

5' CGATGAATTCAGCTTGCAGCAGCCATCAGTAC 3'

PCR reactions utilizing the primers were performed as described above to first screen the cDNA pools of 300,000 clones and then the appropriate subset of the smaller pools of 3000 clones. Three pools of 3000 clones which produced a PCR product of the expected size were then used to transform bacteria.

C. Library Screening by Hybridization

DNA from the transformed bacteria was subsequently screened by hybridization using the original cloned PCR fragment as a probe. Colonies were blotted onto nitrocellulose and prehybridized and hybridized in 50% formamide, 0.75M sodium chloride, 0.075M sodium citrate, 0.05M sodium phosphate pH 6.5, 1% polyvinyl pyrolidine, 1% Ficoll, 1% bovine serum albumin and 50 ng/ml sonicated salmon sperm DNA. The hybridization probe was labeled by random hexamer priming. After overnight hybridization at 42°C, blots were washed extensively in 0.03M sodium chloride, 3mM sodium citrate, 0.1% SDS at 42°C. The nucleotide sequence of 10 hybridizing clones was determined. One of the clones, clone sAH 406-3, contained the sequence predicted by the original peptide sequence of the PAF-AH activity purified from human plasma. The DNA and deduced amino acid sequences of the human plasma PAF-AH are set out in SEQ ID NOs: 7 and 8, respectively.

Clone sAH 406-3 contains a 1.52 kb insert with an open reading frame that encodes a predicted protein of 441 amino acids. At the amino terminus, a relatively hydrophobic segment of 41 residues precedes the N-terminal amino acid (the isoleucine at position 42 of SEQ ID NO: 8) identified by protein microsequencing. The encoded protein may thus have either a long signal sequence or a signal sequence plus an additional peptide that is cleaved to yield the mature functional enzyme. The presence of a signal sequence is one characteristic of secreted proteins. In addition, the protein encoded by clone sAH 406-3 includes the consensus GxSxG motif (amino acids 271-275 of SEQ ID NO: 8) that is believed to contain the active site serine of all known mammalian lipases, microbial lipases and

serine proteases. See Chapus *et al.*, *Biochimie*, *70*: 1223-1224 (1988) and Brenner, *Nature*, *334*: 528-530 (1988).

Table 2 below is a comparison of the amino acid composition of the human plasma PAF-AH of the invention as predicted from SEQ ID NO: 8 and the amino acid composition of the purportedly purified material described by Stafforini *et al.* (1987), *supra*.

Table 2

	Clone sAH 406-3	Stafforini *et al.*
Ala	26	24
Asp & Asn	48	37
Cys	5	14
Glu & Gln	36	42
Phe	22	12
Gly	29	58
His	13	24
Ile	31	17
Lys	26	50
Leu	40	26
Met	10	7
Pro	15	11
Arg	18	16
Ser	27	36
Thr	20	15
Val	13	14
Trp	7	Not determined
Tyr	14	13

The amino acid composition of the mature form of the human plasma PAF-AH of the invention and the amino acid composition of the previously purified material that was purportedly the human plasma PAF-AH are clearly distinct.

When alignment of the Hattori *et al.*, *supra* nucleotide and deduced amino acid sequences of bovine brain cytoplasmic PAF-AH with the nucleotide and

amino acid sequences of the human plasma PAF-AH of the invention was attempted. no significant structural similarity in the sequences was observed.

Example 4

A putative splice variant of the human PAF-AH gene was detected when PCR was performed on macrophage and stimulated PBMC cDNA using primers that hybridized to the 5' untranslated region (nucleotides 31 to 52 of SEQ ID NO: 7) and the region spanning the translation termination codon at the 3' end of the PAF-AH cDNA (nucleotides 1465 to 1487 of SEQ ID NO: 7). The PCR reactions yielded two bands on a gel, one corresponding to the expected size of the PAF-AH cDNA of Example 3 and the other was about 100 bp shorter. Sequencing of both bands revealed that the larger band was the PAF-AH cDNA of Example 3 while the shorter band lacked exon 2 (Example 5 below) of the PAF-AH sequence which encodes the putative signal and pro-peptide sequences of plasma PAF-AH. The predicted catalytic triad and all cysteines were present in the shorter clone, therefore the biochemical activity of the protein encoded by the clone is likely to match that of the plasma enzyme.

To begin to assess the biological relevance of the PAF-AH splice variant that is predicted to encode a cytoplasmically active enzyme, the relative abundance of the two forms in blood monocyte-derived macrophages was assayed by RNase protection. Neither message was present in freshly isolated monocytes but both messages were found at day 2 of *in vitro* differentiation of the monocytes into macrophages and persisted through 6 days of culture. The quantity of the two messages was approximately equivalent throughout the differentiation period. In contrast, similar analyes of neural tissues revealed that only full length message predicted to encode the full length extracellular form of PAF-AH is expressed.

Example 5

Genomic human plasma PAF-AH sequences were also isolated. The structure of the PAF-AH gene was determined by isolating lambda and P1 phage clones containing human genomic DNA by DNA hybridization under conditions of high stringency. Fragments of the phage clones were subcloned and sequenced using

primers designed to anneal at regular intervals throughout the cDNA clone sAH 406-3. In addition, new sequencing primers designed to anneal to the intron regions flanking the exons were used to sequence back across the exon-intron boundaries to confirm the sequences. Exon/intron boundaries were defined as the points where the genomic and cDNA sequences diverged. These analyses revealed that the human PAF-AH gene is comprised of 12 exons.

Exons 1, 2, 3, 4, 5, 6, and part of 7 were isolated from a male fetal placental library constructed in *lamda* FIX (Stratagene). Phage plaques were blotted onto nitrocellulose and prehybridized and hybridized in 50% formamide, 0.75M sodium chloride, 75mM sodium citrate, 50mM sodium phosphate (pH 6.5), 1% polyvinyl pyrolidine, 1% Ficoll, 1% bovine serum albumin, and 50 ng/ml sonicated salmon sperm DNA. The hybridization probe used to identify a phage clone containing exons 2-6 and part of 7 consisted of the entire cDNA clone sAH 406-3. A clone containing exon 1 was identified using a fragment derived from the 5' end of the cDNA clone (nucleotides 1 to 312 of SEQ ID NO: 7). Both probes were labelled with ^{32}P by hexamer random priming. After overnight hybridization at 42°C, blots were washed extensively in 30mM sodium chloride, 3mM sodium citrate, 0.1% SDS at 42°C. The DNA sequences of exons 1, 2, 3, 4, 5, and 6 along with partial surrounding intron sequences are set out in SEQ ID NOs: 9, 10, 11, 12, 13, and 14, respectively.

The remainder of exon 7 as well as exons 8, 9, 10, 11, and 12 were subcloned from a P1 clone isolated from a human P1 genomic library. P1 phage plaques were blotted onto nitrocellulose and prehybridized and hybridized in 0.75M sodium chloride, 50mM sodium phosphate (pH 7.4), 5mM EDTA, 1% polyvinyl pyrolidine, 1% Ficoll, 1% bovine serum albumin, 0.5% SDS, and 0.1 mg/ml total human DNA. The hybridization probe, labeled with ^{32}P by hexamer random priming, consisted of a 2.6 kb EcoR1 fragment of genomic DNA derived from the 3' end of a lambda clone isolated above. This fragment contained exon 6 and the part of exon 7 present on the phage clone. After overnight hybridization at 65°C, blots were washed as described above. The DNA sequences of exons 7, 8, 9, 10, 11, and 12 along with partial surrounding intron sequences are set out in SEQ ID NOs: 15, 16, 17, 18, 19, and 20, respectively.

Example 6

Full length plasma PAF-AH cDNA clones were isolated from mouse, canine, bovine and chicken spleen cDNA libraries and a partial rodent clone was isolated from a rat thymus cDNA library. The clones were identified by low stringency hybridization to the human cDNA (hybridization conditions were the same as described for exons 1 through 6 in Example 5 above except that 20% formamide instead of 50% formamide was used). A 1 kb HindIII fragment of the human PAF-AH sAH 406-3 cDNA clone (nucleotides 309 to 1322 of SEQ ID NO: 7) was used as a probe. In addition, a partial monkey clone was isolated from macaque brain cDNA by PCR using primers based on nucleotides 285 to 303 and 851 to 867 of SEQ ID NO: 7. The nucleotide and deduced amino acid sequences of the mouse, canine, bovine, chicken, rat, and macaque cDNA clones are set out in SEQ ID NOs: 21, 22, 23, 24, 25, and 26, respectively.

A comparison of the deduced amino acid sequences of the cDNA clones with the human cDNA clone results in the amino acid percentage identity values set out in Table 3 below.

Table 3

	Human	Dog	Mouse	Bovine	Chicken
Dog	80	100	64	82	50
Mouse	66	64	100	64	47
Monkey	92	82	69	80	52
Rat	74	69	82	69	55
Bovine	82	82	64	100	50
Chicken	50	50	47	50	100

About 38% of the residues are completely conserved in all the sequences. The most divergent regions are at the amino terminal end (containing the signal sequence) and the carboxyl terminal end which are shown in Example 10 as not critical for enzymatic activity. The Gly-Xaa-Ser-Xaa-Gly motif (SEQ ID NO: 27) found in neutral lipases and other esterases was conserved in the bovine, canine, mouse, rat and chicken PAF-AH. The central serine of this motif serves as the active

site nucleophile for these enzymes. The predicted aspartate and histidine components of the active site (Example 10A) were also conserved. The human plasma PAF-AH of the invention therefore appears to utilize a catalytic triad and may assume the α/β hydrolase conformation of the neutral lipases even though it does not exhibit other sequence homology to the lipases.

Moreover, human plasma PAF-AH is expected to have a region that mediates its specific interaction with the low density and high density lipoprotein particles of plasma. Interaction with these particles may be mediated by the N-terminal half of the molecule which has large stretches of amino acids highly conserved among species but does not contain the catalytic triad of the enzyme.

Example 7

To determine whether human plasma PAF-AH cDNA clone sAH 406-3 (Example 3) encodes a protein having PAF-AH activity, the pRc/CMV expression construct was transiently expressed in COS 7 cells. Three days following transfection by a DEAE Dextran method, COS cell media was assayed for PAF-AH activity.

Cells were seeded at a density of 300,000 cells per 60 mm tissue culture dish. The following day, the cells were incubated in DMEM containing 0.5 mg/ml DEAE dextran, 0.1mM chloroquine and 5-10 μg of plasmid DNA for 2 hours. Cells were then treated with 10% DMSO in phosphate-buffered saline for 1 minute, washed with media and incubated in DMEM containing 10% fetal calf serum previously treated with diisopropyl fluorophosphate (DFP) to inactivate endogenous bovine serum PAF-AH. After 3 days of incubation, media from transfected cells were assayed for PAF-AH activity. Assays were conducted in the presence and absence of either 10 mM EDTA or 1 mM DFP to determine whether the recombinant enzyme was calcium-independent and inhibited by the serine esterase inhibitor DFP as previously described for plasma PAF-AH by Stafforini *et al.* (1987), *supra*. Negative controls included cells transfected with pRc/CMV either lacking an insert or having the sAH 406-3 insert in reverse orientation.

PAF-AH activity in transfectant supernatants was determined by the method of Stafforini *et al.* (1990), *supra,* with the following modifications. Briefly, PAF-AH activity was determined by measuring the hydrolysis of ^{3}H-acetate from

[acetyl-^{3}H] PAF (New England Nuclear, Boston, MA). The aqueous free ^{3}H-acetate was separated from labeled substrate by reversed-phase column chromatography over octadecylsilica gel cartridges (Baker Research Products, Phillipsburg, PA). Assays were carried out using 10 μl transfectant supernatant in 0.1M Hepes buffer, pH 7.2, in a reaction volume of 50 μl. A total of 50 pmoles of substrate were used per reaction with a ratio of 1:5 labeled: cold PAF. Reactions were incubated for 30 minutes at 37°C and stopped by the addition of 40 μl of 10M acetic acid. The solution was then washed through the octadecylsilica gel cartridges which were then rinsed with 0.1M sodium acetate. The aqueous eluate from each sample was collected and counted in a liquid scintillation counter for one minute. Enzyme activity was expressed in counts per minute.

As shown in FIGURE 2, media from cells transfected with sAH 406-3 contained PAF-AH activity at levels 4-fold greater than background. This activity was unaffected by the presence of EDTA but was abolished by 1mM DFP. These observations demonstrate that clone sAH 406-3 encodes an activity consistent with the human plasma enzyme PAF-AH.

Example 8

Human plasma PAF-AH cDNA was expressed in *E. coli* and yeast and stably expressed in mammalian cells by recombinant methods.

A. Expression in *E. coli*

PCR was used to generate a protein coding fragment of human plasma PAF-AH cDNA from clone sAH 406-3 which was readily amenable to subcloning into an *E. coli* expression vector. The subcloned segment began at the 5′ end of the human gene with the codon that encodes Ile_{42} (SEQ ID NO: 8), the N-terminal residue of the enzyme purified from human plasma. The remainder of the gene through the native termination codon was included in the construct. The 5′ sense PCR primer utilized was:

SEQ ID NO: 28

5′ TATTCTAGAATTATGATACAAGTATTAATGGCTGCTGCAAG 3′

and contained an *Xba*I cloning site as well as a translation initiation codon (underscored). The 3′ antisense primer utilized was:

SEQ ID NO: 29

5' ATTGATATCCTAATTGTATTTCTCTATTCCTG 3'

and encompassed the termination codon of sAH 406-3 and contained an *Eco*RV cloning site. PCR reactions were performed essentially as described in Example 3. The resulting PCR product was digested with *Xba*I and *Eco*RV and subcloned into a pBR322 vector containing the Trp promoter [deBoer *et al.*, *PNAS*, *80*:21-25 (1983)] immediately upstream of the cloning site. *E. coli* strain XL-1 Blue was transformed with the expression construct and cultured in L broth containing 100 μg/ml of carbenicillin. Transformants from overnight cultures were pelleted and resuspended in lysis buffer containing 50mM Tris-HCl pH 7.5, 50mM NaCl, 10mM CHAPS, 1mM EDTA, 100 μg/ml lysozyme, and 0.05 trypsin-inhibiting units (TIU)/ml Aprotinin. Following a 1 hour incubation on ice and sonication for 2 minutes, the lysates were assayed for PAF-AH activity by the method described in Example 4. *E. coli* transformed with the expression construct (designated trp AH) generated a product with PAF-AH activity. See Table 6 in Example 9.

Constructs including three additional promoters, the *tacII* promoter (deBoer, *supra*), the arabinose (*ara*) B promoter from *Salmonella typhimurium* [Horwitz *et al.*, *Gene*, *14*: 309-319 (1981)], and the bacteriophage T7 promoter, were also utilized to drive expression of human PAF-AH sequences in *E. coli*. Constructs comprising the Trp promoter (pUC trp AH), the *tacII* promoter (pUC tac AH), and the *ara*B promoter (pUC ara AH) were assembled in plasmid pUC19 (New England Biolabs, MA) while the construct comprising the T7 promoter (pET AH) was assembled in plasmid pET15B (Novagen, Madison, WI). A construct containing a hybrid promoter, pHAB/PH, consisting of the *ara*B promoter fused to the ribosome binding sites of the T7 promoter region was also assembled in pET15B. All *E. coli* constructs produced PAF-AH activity within a range of 20 to 50 U/ml/OD_{600}. This activity corresponded to a total recombinant protein mass of $\geq 1\%$ of the total cell protein.

Several *E. coli* expression constructs were also evaluated which produce PAF-AH with extended amino termini. The N-terminus of natural plasma PAF-AH was identified as Ile_{42} by amino acid sequencing (Example 2). However, the sequence immediately upstream of Ile_{42} does not conform to amino acids found

at signal sequence cleavage sites [*i.e.*, the "-3-1-rule" is not followed, as lysine is not found at position -1; see von Heijne, *Nuc. Acids Res.*, *14*:4683-4690 (1986)]. Presumably a more classical signal sequence (M_1 -A_{17}) is recognized by the cellular secretion system, followed by endoproteolytic cleavage. The entire coding sequence for PAF-AH beginning at the initiating methionine (nucleotides 162 to 1487 of SEQ ID NO: 7) was engineered for expression in *E. coli* using the *trp* promoter. As shown in Table 4, this construct made active PAF-AH, but expression was at about one fiftieth of the level of the original construct beginning at Ile_{42}. Another expression construct, beginning at Val_{18} (nucleotides 213 to 1487 of SEQ ID NO: 7), produced active PAF-AH at about one third the level of the original construct. These results suggest that amino terminal end extensions are not critical or necessary for activity of recombinant PAF-AH produced in *E. coli*.

Table 4

	PAF-AH activity (U/ml/OD_{600})	
Construct	Lysate	Media
pUC trp AH	177.7	0.030
pUC trp AH Met_1	3.1	0.003
pUC trp AH Val_{18}	54.6	0.033

Recombinant human PAF-AH was also produced in *E. coli* using a low copy number plasmid and a promoter that can be induced by the addition of arabinose to the culture. The PAF-AH protein encoded within the plasmid begins at the methionine forty-six residues from the N-terminus of the polypeptide encoded by full length PAF-AH cDNA.

The plasmid used for production of human PAF-AH in bacterial cells was pBAR2/PH.2, which is a pBR322-based plasmid that carries (1) nucleotides 297 to 1487 of SEQ ID NO: 7 encoding human PAF-AH beginning with the methionine codon at position 46, (2) the araB-C promoters and araC gene from the arabinose operon of *Salmonella typhimurium*, (3) a transcription termination sequence from the bacteriophage T7, and (4) a replication origin from bacteriophage f1.

Specifically, pBAR2/PH.2 included the following segments of DNA: (1) from the destroyed AatII site at position 1994 to the EcoRI site at nucleotide 6274, vector sequence containing an origin of replication and genes encoding resistance to either ampicillin or tetracycline derived from the bacterial plasmid pBR322; (2) from the EcoRI site at position 6274 to the XbaI site at position 131, DNA from the *Salmonella typhimurium* arabinose operon (Genbank accession numbers M11045, M11046, M11047, J01797); (3) from the XbaI site at position 131 to the NcoI site at position 170, DNA containing a ribosome binding site from pET-21b (Novagen, Madison, WI); (4) from the NcoI site at position 170 to the XhoI site at position 1363, human PAF-AH cDNA sequence; and (5) from the XhoI site at position 1363 to the destroyed AatII site at position 1993, a DNA fragment from pET-21b (Novagen) that contains a transcription termination sequence from bacteriophae T7 and an origin of replication from bacteriophage f1.

Expression of PAF-AH in pBAR2/PH.2 is under the control of the araB promoter, which is tightly repressed in the presence of glucose and absence of arabinose, but functions as a strong promoter when L-arabinose is added to cultures depleted of glucose. Selection for cells containing the plasmid can be accomplished through the addition of either ampicillin (or related antibiotics) or tetracycline to the culture medium.

The *E. coli* strain used for production of PAF-AH is MC1061 (ATCC 53338), which carries a deletion of the arabinose operon and thereby cannot metabolize arabinose. The advantage of using a strain that is unable to break down arabinose is that the inducer (arabinose) for production of PAF-AH is not depleted from the medium during the induction period, resulting in higher levels of PAF-AH compared to that obtained with strains that are capable of metabolizing arabinose. MC1061 is also a leucine auxotroph and was cultivated by batch-fed process using a defined media containing casamino acids that complement the leucine mutation. Cells were grown at 30°C in batch media containing 2 gm/L glucose. Glucose serves the dual purpose of carbon source for cell growth, and repressor of the arabinose promoter. When batch glucose levels were depleted (<50 mg/L), a nutrient feed (containing 300 gm/L glucose) was started. The feed was increased linearly for 16 hours at a rate which limited acid bi-product formation. At this point, the nutrient feed was

switched to media containing glycerol instead of glucose. Simultaneously, 500 gm/L L-arabinose was added to a final concentration of 5 gm/L. The glycerol feed was kept at a constant feed rate for 22 hours. Cells were harvested using hollow-fiber filtration to concentrate the suspension approximately 10-fold. Cell paste was stored at -70°C. A final cell mass of about 80 gm/L was obtained (OD_{600} = 50-60) with a PAF-AH activity of 65-70 U/OD/ml representing about 10% of total cell protein. The final culture volume of about 75 liters contained 50-60 gm PAF-AH.

B. Expression in Yeast Cells

Recombinant human PAF-AH was also expressed in *Saccharomyces cerevisiae*. The yeast ADH2 promoter was used to drive rPAF-AH expression and produced 7 U/ml/OD_{600} (Table 5 below).

Table 5

Construct	Promoter	Strain	Enzyme Activity (U/ml/OD)
pUC tac AH	*tac*	*E. coli* W3110	30
pUC trp AH	*trp*	*E. coli* W3110	40
pUC ara AH	*ara*B	*E. coli* W3110	20
pET AH	T7	*E. coli* BL21 (DE3) (Novagen)	50
pHAB/PH	*ara*B/T7	*E. coli* XL-1	34
pBAR2/PH.2	*ara*B	MC1061	90
pYep ADH2 AH	ADH2	Yeast BJ2.28	7

C. Expression of PAF-AH in mammalian cells

1. Expression of Human PAF-AH cDNA Constructs

Plasmids constructed for expression of PAF-AH, with the exception of pSFN/PAFAH.1, employ a strong viral promoter from cytomegalovirus, a polyadenylation site from the bovine growth hormone gene, and the SV40 origin of

replication to permit high copy number replication of the plasmid in COS cells. Plasmids were electroporated into cells.

A first set of plasmids was constructed in which the 5' flanking sequence (pDC1/PAFAH.1) or both the 5' or 3' flanking sequences (PDC1/PAFAH.2) of the human PAF-AH cDNA were replaced with flanking sequences from other genes known to be expressed at high levels in mammalian cells. Transfection of these plasmids into COS, CHO or 293 cells led to production of PAF-AH at about the same level (0.01 units/ml or 2-4 fold above background) as that cited for clone sAH 406-3 in Example 7 after transient transfection of COS cells. Another plasmid was constructed which included a Friend spleen focus-forming virus promoter instead of the cytomegalovirus promoter. The human PAF-AH cDNA was inserted into plasmid pmH-neo [Hahn *et al.*, *Gene*, *127*: 267 (1993)] under control of the Friend spleen focus-forming virus promoter. Transfection of the myeloma cell line NS0 with the plasmid which was designated pSFN/PAFAH.1 and screening of several hundred clones resulted in the isolation of two transfectants (4B11 and 1C11) that made 0.15-0.5 units/ml of PAF-AH activity. Assuming a specific activity of 5000 units/milligram, the productivity of these two NS0 transfectants corresponds to about 0.1 mg/liter.

2. Expression of Mouse-Human Chimeric PAF-AH Gene Constructs

A construct (pRc/MS9) containing the cDNA encoding mouse PAF-AH in the mammalian expression vector pRc/CMV resulted in production of secreted PAF-AH at the level of 5-10 units/ml (1000 fold above background) after transfection into COS cells. Assuming that the specific activity of the mouse PAF-AH is about the same as that of the human enzyme, the mouse cDNA is therefore expressed at a 500-1000 fold higher level than is the human PAF-AH cDNA.

To examine the difference between the expression levels of human and mouse PAF-AH in COS cells, two mouse-human chimeric genes were constructed and tested for expression in COS cells. The first of these constructs, pRc/PH.MHC1, contains the coding sequence for the N-terminal 97 amino acids of the mouse PAF-AH polypeptide (SEQ ID NO: 21) fused to the C-terminal 343 amino acids of human PAF-AH in the expression vector pRc/CMV (Invitrogen, San Diego, CA). The second chimeric gene, in plasmid pRc/PH.MHC2, contains the coding sequence for

the N-terminal 40 amino acids of the mouse PAF-AH polypeptide fused to the C-terminal 400 residues of human PAF-AH in pRc/CMV. Transfection of COS cells with pRc/PH.MHC1 led to accumulation of 1-2 units/ml of PAF-AH activity in the media. Conditioned media derived from cells transfected with pRc/PH.MHC2 was found to contain only 0.01 units/ml of PAF-AH activity. From these experiments, it appears that the difference in expression level between mouse and human PAF-AH genes is attributable at least in part to the polypeptide segment between the residues 40 and 97, or the corresponding RNA or DNA segment encoding this region of the PAF-AH protein.

3. Recoding of the First 290 bp of the PAF-AH Coding Sequence

One hypothesis for the low level of human PAF-AH synthesized in transfected mammalian cells is that the codons utilized by the natural gene are suboptimal for efficient expression. However, it does not seem likely that codon usage can account for 500-1000 fold difference in expression levels between the mouse and human genes because optimizing codons generally has at most only a 10-fold effect on expression. A second hypothesis to explain the difference between the mouse and human PAF-AH expression levels is that the human PAF-AH mRNA in the 5' coding region forms a secondary structure that leads to either relatively rapid degradation of the mRNA or causes inefficient translation initiation or elongation.

To test these hypotheses, a synthetic fragment encoding the authentic human PAF-AH protein from the amino-terminus to residue 96 but in which most of the codons have been substituted ("recoded") with a codon of a different sequence but encoding the same amino acid was constructed. Changing the second codon from GTG to GTA resulted in the creation of an Asp718 site, which was at one end of the synthetic fragment and which is present in the mouse cDNA. The other end of the fragment contained the BamHI site normally found at codon 97 of the human gene. The approximately 290 bp Asp718/BamHI fragment was derived from a PCR fragment that was made using the dual asymmetric PCR approach for construction of synthetic genes described in Sandhu *et al.*, *Biotechniques*, *12*: 14-16 (1992). The synthetic Asp718/BamHI fragment was ligated with DNA fragments encoding the remainder of the human PAF-AH molecule beginning with nucleotide 453 of SEQ ID NO: 7 such that a sequence encoding authentic human PAF-AH enzyme was inserted

into the mammalian expression vector pRc/CMV (Invitrogen, San Diego) to create plasmid pRc/HPH.4. The complete sequence of the recoded gene is set out in SEQ ID NO: 30. The 5' flanking sequence adjacent to the human PAF-AH coding sequence in pRc/HPH.4 is from that of a mouse cDNA encoding PAF-AH in pRc/MS9 (nucleotides 1 to 116 of SEQ ID NO: 21).

To test expression of human PAF-AH from pRc/HPH.4, COS cells were transiently transfected with pRc/HPH.4 (recoded human gene), pRc/MS9 (mouse PAF-AH), or pRc/PH.MHC1 (mouse-human hybrid 1). The conditioned media from the transfected cells were tested for PAF-AH activity and found to contain 5.7 units/ml (mouse gene), 0.9 units/ml (mouse-human hybrid 1), or 2.6 units/ml (recoded human gene). Thus, the strategy of recoding the first 290 bp of coding sequence of human PAF-AH was successful in boosting expression levels of human PAF-AH from a few nanograms/ml to about 0.5 microgram/ml in a transient COS cell transfection. The recoded PAF-AH gene from pRc/HPH.4 will be inserted into a mammalian expression vector containing the dihydrofolate reductase (DHFR) gene and DHFR-negative chinese hamster ovary cells will be transfected with the vector. The transfected cells will be subjected to methotrexate selection to obtain clones making high levels of human PAF-AH due to gene amplification.

Example 9

Recombinant human plasma PAF-AH (beginning at Ile_{42}) expressed in *E. coli* was purified to a single Coomassie-stained SDS-PAGE band by various methods and assayed for activities exhibited by the native PAF-AH enzyme.

A. Purification of Recombinant PAF-AH

The first purification procedure utilized is similar to that described in Example 1 for native PAF-AH. The following steps were performed at 4°C. Pellets from 50 ml PAF-AH producing *E. coli* (transformed with expression construct trp AH) were lysed as described in Example 8. Solids were removed by centrifugation at 10,000 g for 20 minutes. The supernatant was loaded at 0.8 ml/minute onto a Blue Sepharose Fast Flow column (2.5 cm x 4 cm; 20 ml bed volume) equilibrated in buffer D (25mM Tris-HCl, 10mM CHAPS, 0.5M NaCl, pH 7.5). The column was washed with 100 ml buffer D and eluted with 100 ml buffer A containing 0.5M

KSCN at 3.2 ml/minute. A 15 ml active fraction was loaded onto a 1 ml Cu Chelating Sepharose column equilibrated in buffer D. The column was washed with 5 ml buffer D followed by elution with 5 ml of buffer D containing 100mM imidazole with gravity flow. Fractions containing PAF-AH activity were analyzed by SDS-PAGE.

The results of the purification are shown in Table 6 wherein a unit equals μmol PAF hydrolysis per hour. The purification product obtained at 4°C appeared on SDS-PAGE as a single intense band below the 43 kDa marker with some diffuse staining directly above and below it. The recombinant material is significantly more pure and exhibits greater specific activity when compared with PAF-AH preparations from plasma as described in Example 1.

Table 6

Sample	Volume (ml)	Activity (units/ ml)	Total Act. (units x 10^3)	Prot Conc (mg/mL)	Specific Activity (units/ mg)	% Recovery of Activity Step	Cum.	Fold Purification Step	Cum.
Lysate	4.5	989	4451	15.6	63	100	100	1	1
Blue	15	64	960	0.07	914	22	22	14.4	14.4
Cu	1	2128	2128	0.55	3869	220	48	4.2	61

When the same purification protocol was performed at ambient temperature, in addition to the band below the 43 kDa marker, a group of bands below the 29 kDa marker correlated with PAF-AH activity of assayed gel slices. These lower molecular weight bands may be proteolytic fragments of PAF-AH that retain enzymatic activity.

A different purification procedure was also performed at ambient temperature. Pellets (100 g) of PAF-AH-producing *E. coli* (transformed with the expression construct pUC trp AH) were resuspended in 200 ml of lysis buffer (25mM Tris, 20mM CHAPS, 50mM NaCl, 1mM EDTA, 50 μg/ml benzamidine, pH 7.5) and lysed by passing three times through a microfluidizer at 15,000 psi. Solids were

removed by centrifugation at 14,300 x g for 1 hour. The supernatant was diluted 10-fold in dilution buffer [25mM MES (2-[N-morpholino] ethanesulfonic acid), 10mM CHAPS, 1mM EDTA, pH 4.9] and loaded at 25 ml/minute onto an S Sepharose Fast Flow Column (200 ml) (a cation exchange column) equilibrated in Buffer E (25mM MES, 10mM CHAPS, 1mM EDTA, 50mM NaCl, pH 5.5). The column was washed with 1 liter of Buffer E, eluted with 1M NaCl, and the eluate was collected in 50 ml fractions adjusted to pH 7.5 with 0.5 ml of 2M Tris base. Fractions containing PAF-AH activity were pooled and adjusted to 0.5M NaCl. The S pool was loaded at 1 ml/minute onto a Blue Sepharose Fast Flow column (2.5 cm x 4 cm; 20 ml) equilibrated in Buffer F (25mM Tris, 10mM CHAPS, 0.5M NaCl, 1mM EDTA, pH 7.5). The column was washed with 100 ml Buffer F and eluted with 100 ml Buffer F containing 3M NaCl at 4 ml/minute. The Blue Sepharose Fast Flow chromatography step was then repeated to reduce endotoxin levels in the sample. Fractions containing PAF-AH activity were pooled and dialyzed against Buffer G (25mM Tris pH 7.5, 0.5M NaCl, 0.1% Tween 80, 1mM EDTA).

The results of the purification are shown in Table 7 wherein a unit equals μmol PAF hydrolysis per hour.

Table 7

Sample	Volume (ml)	Activity (units/ ml)	Total Act. (units x 10^3)	Prot Conc (mg/mL)	Specific Activity (units/ mg)	% Recovery of Activity Step	Cum.	Fold Purification Step	Cum.
Lysate	200	5640	1128	57.46	98	100	100	1	1
S	111	5742	637	3.69	1557	57	56	16	16
Blue	100	3944	394	0.84	4676	35	62	3	48

The purification product obtained appeared on SDS-PAGE as a single intense band below the 43 kDa marker with some diffuse staining directly above and below it. The recombinant material is significantly more pure and exhibits greater specific activity when compared with PAF-AH preparations from plasma as described in Example 1.

Yet another purification procedure contemplated by the present invention involves the following cell lysis, clarification, and first column steps. Cells are diluted 1:1 in lysis buffer (25mM Tris pH 7.5, 150mM NaCl, 1% Tween 80, 2mM EDTA). Lysis is performed in a chilled microfluidizer at 15,000-20,000 psi with three passes of the material to yield >99% cell breakage. The lysate is diluted 1:20 in dilution buffer (25mM Tris pH 8.5, 1mM EDTA) and applied to a column packed with Q-Sepharose Big Bead chromatography media (Pharmacia) and equilibrated in 25mM Tris pH 8.5, 1mM EDTA, 0.015% Tween 80. The eluate is diluted 1:10 in 25mM MES pH 5.5, 1.2M Ammonium sulfate, 1mM EDTA and applied to Butyl Sepharose chromography media (Pharmacia) equilibrated in the same buffer. PAF-AH activity is eluted in 25mM MES pH. 5.5, 0.1% Tween 80, 1mM EDTA.

Still another method contemplated by the invention for purifying enzymatically-active PAF-AH from *E.coli* includes the steps of: (a) preparing an *E.coli* extract which yields solubilized PAF-AH supernatant after lysis in a buffer containing CHAPS; (b) dilution of the said supernatant and application to a anion exchange column equilibrated at about pH 8.0; (c) eluting PAF-AH enzyme from said anion exchange column; (d) applying said adjusted eluate from said anion exchange column to a blue dye ligand affinity column; (e) eluting the said blue dye ligand affinity column using a buffer comprising 3.0M salt; (f) dilution of the blue dye eluate into a suitable buffer for performing hydroxylapatite chromatography; (g) performing hydroxylapatite chromatography where washing and elution is accomplished using buffers (with or without CHAPS); (h) diluting said hydroxylapatite eluate to an appropriate salt concentration for cation exchange chromatography; (i) applying said diluted hydroxylapatite eluate to a cation exchange column at a pH ranging between approximately 6.0 to 7.0; (j) elution of PAF-AH from said cation exchange column with a suitable formulation buffer; (k) performing cation exchange chromatography in the cold; and (l) formulation of PAF-AH in liquid or frozen form in the absence of CHAPS.

Preferably in step (a) above the lysis buffer is 25mM Tris, 100mM NaCl, 1mM EDTA, 20mM CHAPS, pH 8.0; in step (b) the dilution of the supernatant for anion exchange chromatography is 3-4 fold into 25mM Tris, 1mM EDTA, 10mM

CHAPS, pH 8.0 and the column is a Q-Sepharose column equilibrated with 25mM Tris, 1mM EDTA, 50mM NaCl, 10mM CHAPS, pH 8.0; in step (c) the anion exchange column is eluted using 25mM Tris, 1mM EDTA, 350mM NaCl, 10mM CHAPS, pH 8.0; in step (d) the eluate from step (c) is applied directly onto a blue dye affinity column; in step (e) the column is eluted with 3M NaCl, 10mM CHAPS, 25mM Tris, pH 8.0 buffer; in step (f) dilution of the blue dye eluate for hydroxylapatite chromatography is accomplished by dilution into 10mM sodium phosphate, 100mM NaCl, 10mM CHAPS, pH 6.2; in step (g) hydroxylapatite chromatography is accomplished using a hydroxylapatite column equilibrated with 10mM sodium phosphate, 100mM NaCl, 10mM CHAPS and elution is accomplished using 50mM sodium phosphate, 100mM NaCl (with or without) 10mM CHAPS, pH 7.5; in step (h) dilution of said hydroxylapatite eluate for cation exchange chromatography is accomplished by dilution into a buffer ranging in pH from approximately 6.0 to 7.0 comprising sodium phosphate (with or without CHAPS); in step (i) a S Sepharose column is equilibrated with 50mM sodium phosphate, (with or without) 10mM CHAPS, pH 6.8; in step (j) elution is accomplished with a suitable formulation buffer such as potassium phosphate 50mM, 12.5mM aspartic acid, 125mM NaCl, pH 7.5 containing 0.01% Tween-80; and in step (k) cation exchange chromatrography is accomplished at 2-8°C. Examples of suitable formulation buffers for use in step (l) which stabilize PAF-AH include 50mM potassium phosphate, 12.5mM Aspartic acid, 125mM NaCl pH 7.4 (approximately, with and without the addition of Tween-80 and or Pluronic F68) or 25mM potassium phosphate buffer containing (at least) 125mM NaCl, 25mM arginine and 0.01% Tween-80 (with or without Pluronic F68 at approximately 0.1 and 0.5%).

B. Activity of Recombinant PAF-AH

The most remarkable property of the PAF acetylhydrolase is its marked specificity for substrates with a short residue at the *sn*-2 position of the substrate. This strict specificity distinguishes PAF acetylhydrolase from other forms of PLA_2. Thus, to determine if recombinant PAF-AH degrades phospholipids with long-chain fatty acids at the *sn*-2 position, hydrolysis of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (arachidonoylPC) was assayed since this is the preferred substrate for a well-characterized form of PLA_2. As predicted from previous studies with native

PAF-AH, this phospholipid was not hydrolyzed when incubated with recombinant PAF-AH. In additional experiments, arachidonoylPC was included in a standard PAF hydrolysis assay at concentrations ranging from 0 to 125 μM to determine whether it inhibited the hydrolysis of PAF by recombinant PAF-AH. There was no inhibition of PAF hydrolysis even at the highest concentration of PAF-AH, which was 5-fold greater than the concentration of PAF. Thus, recombinant PAF-AH exhibits the same substrate selectivity as the native enzyme; long chain substrates are not recognized. Moreover, recombinant PAF-AH enzyme rapidly degraded an oxidized phospholipid (glutaroylPC) which had undergone oxidative cleavage of the *sn*-2 fatty acid. Native plasma PAF-AH has several other properties that distinguish it from other phospholipases including calcium-independence and resistance to compounds that modify sulfhydryl groups or disrupt disulfides.

Both the native and recombinant plasma PAF-AH enzymes are sensitive to DFP, indicating that a serine comprises part of their active sites. An unusual feature of the native plasma PAF acetylhydrolase is that it is tightly associated with lipoproteins in circulation, and its catalytic efficiency is influenced by the lipoprotein environment. When recombinant PAF-AH of the invention was incubated with human plasma (previously treated with DFP to abolish the endogenous enzyme activity), it associated with low and high density lipoproteins in the same manner as the native activity. This result is significant because there is substantial evidence that modification of low density lipoproteins is essential for the cholesterol deposition observed in atheromas, and that oxidation of lipids is an initiating factor in this process. PAF-AH protects low density lipoproteins from modification under oxidizing conditions *in vitro* and may have such a role *in vivo*. Administration of PAF-AH is thus indicated for the suppression of the oxidation of lipoproteins in atherosclerotic plaques as well as to resolve inflammation.

These results all confirm that the cDNA clone sAH 406-3 encodes a protein with the activities of the the human plasma PAF acetylhydrolase.

Example 10

Various other recombinant PAF-AH products were expressed in *E. coli*. The products included PAF-AH analogs having single amino acid mutations and PAF-AH fragments.

A. PAF-AH Amino Acid Substitution Products

PAF-AH is a lipase because it hydrolyses the phospholipid PAF. While no obvious overall similarity exists between PAF-AH and other characterized lipases, there are conserved residues found in comparisons of structurally characterized lipases. A serine has been identified as a member of the active site. The serine, along with an aspartate residue and a histidine residue, form a catalytic triad which represents the active site of the lipase. The three residues are not adjacent in the primary protein sequence, but structural studies have demonstrated that the three residues are adjacent in three dimensional space. Comparisons of structures of mammalian lipases suggest that the aspartate residue is generally twenty-four amino acids C-terminal to the active site serine. In addition, the histidine is generally 109 to 111 amino acids C-terminal to the active site serine.

By site-directed mutagenesis and PCR, individual codons of the human PAF-AH coding sequence were modified to encode alanine residues and were expressed in *E. coli*. As shown in Table 8 below wherein, for example, the abbreviation "S108A" indicates that the serine residue at position 273 was changed to an alanine, point mutations of Ser_{273}, Asp_{296}, or His_{351} completely destroy PAF-AH activity. The distances between active site residues is similar for PAF-AH (Ser to Asp, 23 amino acids; Ser to His, 78 amino acids) and other lipases. These experiments demonstrate that Ser_{273}, Asp_{296}, and His_{351} are critical residues for activity and are therefore likely candidates for catalytic triad residues. Cysteines are often critical for the functional integrity of proteins because of their capacity to form disulfide bonds. The plasma PAF-AH enzyme contains five cysteines. To determine whether any of the five is critical for enzyme actvity, each cysteine was mutated individually to a serine and the resulting mutants were expressed in *E. coli*. As shown below in Table 8, a significant but not total loss of PAF-AH activity resulted from the conversion of either Cys_{229} r Cys_{291} to serine. Therefore, these cysteines

appear to be necessary for full PAF-AH activity. Other point mutations had little or no effect on PAF-AH catalytic activity. In Table 8, "++++" represent wild type PAF-AH activity of about 40-60 U/ml/OD_{600}, "+++" represents about 20-40 U/ml/OD_{600} activity, "++" represents about 10-20 U/ml/OD_{600} activity, "+" represents 1-10 U/ml/OD_{600} activity, and "-" indicates <1 U/ml/OD_{600} activity.

Table 8

Mutation	PAF-AH activity
Wild type	++++
S108A	++++
S273A	-
D286A	-
D286N	++
D296A	-
D304A	++++
D338A	++++
H351A	-
H395A, H399A	++++
C67S	+++
C229S	+
C291S	+
C334S	++++
C407S	+++

B. PAF-AH Fragment Products

C-terminal deletions were prepared by digesting the 3' end of the PAF-AH coding sequence with exonuclease III for various amounts of time and then ligating the shortened coding sequence to plasmid DNA encoding stop codons in all three reading frames. Ten different deletion constructs were characterized by DNA sequence analysis, protein expression, and PAF-AH activity. Removal of twenty-one to thirty C-terminal amino acids greatly reduced catalytic activity and removal of fifty-two residues completely destroyed activity. See FIGURE 3.

Similar deletions were made at the amino terminal end of PAF-AH. Fusions of PAF-AH with *E. coli* thioredoxin at the N-terminus were prepared to facilitate consistent high level expression PAF-AH activity [LaVallie *et al.*, *Bio/technology*, *11*:187-193 (1993)]. Removal of nineteen amino acids from the naturally processed N-terminus (Ile_{42}) reduced activity by 99% while removal of twenty-six amino acids completely destroyed enzymatic activity in the fusion protein. See FIGURE 3. Deletion of twelve amino acids appeared to enhance enzyme activity about four fold.

In subsequent purifications of PAF-AH from fresh human plasma by a method similar to that described in Example 1 (Microcon 30 filter from Amicon were utilized to concentrate Blue sepharose eluate instead of a Cu column), two N-termini in addition to Ile_{42} were identified, Ser_{35} and Lys_{55}. The heterogeneity may be the natural state of the enzyme in plasma or may occur during purification.

The purified material described above was also subject to analysis for glycosylation. Purified native PAF-AH was incubated in the presence or absence of N-Glycanase, an enzyme that removes N-linked carbohydrates from glycoproteins. The treated PAF-AH samples were electrophoresed through a 12% SDS polyacrylamide gel then visualized by Western blotting using rabbit polyclonal antisera. Protein not treated with N-Glycanase migrated as a diffuse band of 45-50 kDa whereas the protein treated with the glycanase migrated as a tight band of about 44 kDa, demonstrating that native PAF-AH is glycosylated.

Example 11

A preliminary analysis of expression patterns of human plasma PAF-AH mRNA in human tissues was conducted by Northern blot hybridization.

RNA was prepared from human cerebral cortex, heart, kidney, placenta, thymus and tonsil using RNA Stat 60 (Tel-Test "B", Friendswood, TX). Additionally, RNA was prepared from the human hematopoietic precursor-like cell line, THP-1 (ATCC TIB 202), which was induced to differentiate to a macrophage-like phenotype using the phorbol ester phorbolmyristylacetate (PMA). Tissue RNA and RNA prepared from the premyelocytic THP-1 cell line prior to and 1 to 3 days after induction were electrophoresed through a 1.2% agarose formaldehyde gel and subsequently transferred to a nitrocellulose membrane. The full length human plasma PAF-AH cDNA, sAH 406-3, was labelled by random priming and hybridized to the membrane under conditions identical to those described in Example 3 for library screening. Initial results indicate that the PAF-AH probe hybridized to a 1.8 kb band in the thymus, tonsil, and to a lesser extent, the placental RNA.

PAF is synthesized in the brain under normal physiological as well as pathophysiological conditions. Given the known pro-inflammatory and potential neurotoxic properties of the molecule, a mechanism for localization of PAF synthesis or for its rapid catabolism would be expected to be critical for the health of neural tissue. The presence of PAF acetylhydrolase in neural tissues is consistent with it playing such a protective role. Interestingly, both a bovine heterotrimeric intracellular PAF-AH [the cloning of which is described in Hattori *et al.*, *J. Biol. Chem.*, *269*(37): 23150-23155 (1994)] and PAF-AH of the invention have been identified in the brain. To determine whether the two enzymes are expressed in similar or different compartments of the brain, the human homologue of the bovine brain intracellular PAF-AH cDNA was cloned, and its mRNA expression pattern in the brain was compared by Northern blotting to the mRNA expression pattern of the PAF-AH of the invention by essentially the same methods as described in the foregoing paragraph. The regions of the brain examined by Northern blotting were the cerebellum, medulla, spinal cord, putamen, amygdala, caudate nucleus, thalamus, and the occipital pole, frontal lobe and temporal lobe of the cerebral cortex. Message of both enzymes was detected in each of these tissues although the heterotrimeric

heterotrimeric intracellular form appeared in greater abundance than the secreted form. Northern blot analysis of additional tissues further revealed that the heterotrimeric intracellular form is expressed in a broad variety of tissues and cells, including thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocytes, macrophages, brain, liver, skeletal muscle, kidney, pancreas and adrenal gland. This ubiquitous expression suggests that the heterotrimeric intracellular PAF-AH has a general housekeeping function within cells.

The expression of PAF-AH RNA in monocytes isolated from human blood and during their spontaneous differentiation into macrophages in culture was also examined. Little or no RNA was detected in fresh monocytes, but expression was induced and maintained during differentiation into macrophages. There was a concomitant accumulation of PAF-AH activity in the culture medium of the differentiating cells. Expression of the human plasma PAF-AH transcript was also observed in the THP-1 cell RNA at 1 day but not 3 days following induction. THP-1 cells did not express mRNA for PAF-AH in the basal state.

Example 12

PAF-AH expression in human and mouse tissues was examined by *in situ* hybridization.

Human tissues were obtained from National Disease Research Interchange and the Cooperative Human Tissue Network. Normal mouse brain and spinal cord, and EAE stage 3 mouse spinal cords were harvested from S/JLJ mice. Normal S/JLJ mouse embryos were harvested from eleven to eighteen days after fertilization.

The tissue sections were placed in Tissue Tek II cryomolds (Miles Laboratories, Inc., Naperville, IL) with a small amount of OCT compound (Miles, Inc., Elkhart, IN). They were centered in the cryomold, the cryomold filled with OCT compound, then placed in a container with 2-methylbutane [$C_2H_5CH(CH_3)_2$, Aldrich Chemical Company, Inc., Milwaukee, WI] and the container placed in liquid nitrogen. Once the tissue and OCT compound in the cryomold were frozen, the blocks were stored at -80°C until sectioning. The tissue blocks were sectioned at 6 μm thickness and adhered to Vectabond (Vector Laboratories, Inc., Burlingame, CA)

coated slides and stored at -70°C and placed at 50°C for approximately 5 minutes to warm them and remove condensation and were then fixed in 4% paraformaldehyde for 20 minutes at 4°C, dehydrated (70%, 95%, 100% ethanol) for 1 minute at 4°C in each grade, then allowed to air dry for 30 minutes at room temperature. Sections were denatured for 2 minutes at 70°C in 70% formamide/2X SSC, rinsed twice in 2X SSC, dehydrated and then air dried for 30 minutes. The tissues were hybridized *in situ* with radiolabeled single-stranded mRNA generated from DNA derived from an internal 1 Kb HindIII fragment of the PAF-AH gene (nucleotides 308 to 1323 of SEQ ID NO: 7) by *in vitro* RNA transcription incorporation ^{35}S-UTP (Amersham) or from DNA derived from the heterotrimeric intracellular PAF-AH cDNA identified by Hattori *et al.* The probes were used at varying lengths from 250-500 bp. Hybridization was carried out overnight (12-16 hours) at 50°C; the ^{35}S-labeled riboprobes (6 x 10^5 cpm/section), tRNA (0.5 μg/section) and diethylpyrocarbonate (depc)-treated water were added to hybridization buffer to bring it a final concentration of 50% formamide, 0.3M NaCl, 20 mM Tris pH 7.5, 10% dextran sulfate, 1X Denhardt's solution, 100 mM dithiothretol (DTT) and 5 mM EDTA. After hybridization, sections were washed for 1 hour at room temperature in 4X SSC/10 mM DTT, then for 40 minutes at 60°C in 50% formamide/1X SSC/10 mM DTT, 30 minutes at room temperature in 2X SSC, and 30 minutes at room temperature in 0.1X SSC. The sections were dehydrated, air dried for 2 hours, coated with Kodak NTB2 photographic emulsion, air dried for 2 hours, developed (after storage at 4°C in complete darkness) and counterstained with hematoxylin/eosin.

A. Brain

Cerebellum. In both the mouse and the human brains, strong signal was seen in the Purkinje cell layer of the cerebellum, in basket cells, and individual neuronal cell bodies in the dentate nucleus (one of the four deep nuclei in the cerebellum). Message for the intracellular PAF-AH was also observed in these cell types. Additionally, plasma PAF-AH signal was seen on individual cells in the granular and molecular layers of the grey matter.

Hippocampus. In the human hippocampus section, individual cells throughout the section, which appear to be neuronal cell bodies, showed strong

signal. These were identified as polymorphic cell bodies and granule cells. Message for the heterotrimeric intracellular PAF-AH was also observed in hippocampus.

Brain stem. On both human and mouse brain stem sections, there was strong signal on individual cells in the grey matter.

Cortex. On human cortex sections taken from the cerebral, occipital, and temporal cortexes, and on mouse whole brain sections, individual cells throughout the cortex showed strong signal. These cells were identified as pyramidal, stellate and polymorphic cell bodies. There does not appear to be differentiation in the expression pattern in the different layers of the cortex. These *in situ* hybridization results are different from the results for cerebral cortex obtained by Northern blotting. The difference is likely to result from the greater sensitivity of *in situ* hybridization compared to that of Northern blotting. As in the cerebellum and hippocampus, a similar pattern of expression of the heterotrimeric intracellular PAF-AH was observed.

Pituitary. Somewhat weak signal was seen on scattered individual cells in the pars distalis of the human tissue section.

B. Human colon

Both normal and Crohn's disease colons displayed signal in the lymphatic aggregations present in the mucosa of the sections, with the level of signal being slightly higher in the section from the Crohn's disease patient. The Crohn's disease colon also had strong signal in the lamina propria. Similarly, a high level of signal was observed in a diseased appendix section while the normal appendix exhibited a lower but still detectable signal. The sections from the ulcerative colitis patient showed no evident signal in either the lymphatic aggregations or the lamina propria.

C. Human tonsil and thymus

Strong signal was seen on scattered groups of individual cells within the germinal centers of the tonsil and within the thymus.

D. Human lymph node

Strong signal was observed on the lymph node section taken from a normal donor, while somewhat weak signal was observed in the lymph nodules of the section from a donor with septic shock.

E. Human small intestine

Both normal and Crohn's disease small intestine had weak signal in the Peyer's patches and lamina propria in the sections, with the signal on the diseased tissue slightly higher.

F. Human spleen and lung

Signal was not observed on any of the spleen (normal and splenic abcess sections) or lung (normal and emphysema sections) tissues.

G. Mouse spinal cord

In both the normal and EAE stage 3 spinal cords, there was strong signal in the grey matter of the spinal cord, with the expression being slightly higher in the EAE stage 3 spinal cord. In the EAE stage 3 spinal cord, cells in the white matter and perivascular cuffs, probably infiltrating macrophages and/or other leukocytes, showed signal which was absent in the normal spinal cord.

F. Mouse embryos

In the day 11 embryo signal was apparent in the central nervous system in the fourth ventricle, which remained constant throughout the embryo time course as it developed into the cerebellum and brain stem. As the embryos matured, signal became apparent in central nervous system in the spinal cord (day 12), primary cortex and ganglion Gasseri (day 14), and hypophysis (day 16). Signal was observed in the peripheral nervous system (beginning on day 14 or 15) on nerves leaving the spinal cord, and, on day 17, strong signal appeared around the whiskers of the embryo. Expression was also seen in the liver and lung at day 14, the gut (beginning on day 15), and in the posterior portion of the mouth/throat (beginning on day 16). By day 18, the expression pattern had differentiated into signal in the cortex, hindbrain (cerebellum and brain stem), nerves leaving the lumbar region of the spinal cord, the posterior portion of the mouth/throat, the liver, the kidney, and possible weak signal in the lung and gut.

G. Summary

PAF-AH mRNA expression in the tonsil, thymus, lymph node, Peyer's patches, appendix, and colon lymphatic aggregates is consistent with the conclusions that the probable predominant *in vivo* source of PAF-AH is the macrophage because

these tisues all are populated with tissue macrophages that serve as phagocytic and antigen-processing cells.

Expression of PAF-AH in inflamed tissues would be consistent with the hypothesis that a role of monocyte-derived macrophages is to resolve inflammation. PAF-AH would be expected to inactivate PAF and the pro-inflammatory phospholipids, thus down-regulating the inflammatory cascade of events initiated by these mediators.

PAF has been detected in whole brain tissue and is secreted by rat cerebellar granule cells in culture. *In vitro* and *in vivo* experiments have demonstrated that PAF binds a specific receptor in neural tissues and induces functional and phenotypic changes such as calcium mobilization, upregulation of transcription activating genes, and differentiation of the neural precursor cell line, PC12. These observations suggested a physiologic role for PAF in the brain, and consistent with this, recent experiments using hippocampal tissue section cultures and PAF analogs and antagonists have implicated PAF as an important retrograde messenger in hippocampal long term potentiation. Therefore, in addition to its pathological effect in inflammation, PAF appears to participate in routine neuronal signalling processes. Expression of the extracellular PAF-AH in the brain may serve to regulate the duration and magnitude of PAF-mediated signalling.

Example 13

Monoclonal antibodies specific for recombinant human plasma PAF-AH were generated using *E. coli* produced PAF-AH as an immunogen.

Mouse #1342 was injected on day 0, day 19, and day 40 with recombinant PAF-AH. For the prefusion boost, the mouse was injected with the immunogen in PBS, four days later the mouse was sacrificed and its spleen removed sterilely and placed in 10ml serum free RPMI 1640. A single-cell suspension was formed by grinding the spleen between the frosted ends of two glass microscope slides submerged in serum free RPMI 1640, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin (RPMI) (Gibco, Canada). The cell suspension was filtered through sterile 70-mesh Nitex cell strainer (Becton Dickinson, Parsippany, New Jersey), and washed twice by

centrifuging at 200 g for 5 minutes and resuspending the pellet in 20 ml serum free RPMI. Thymocytes taken from 3 naive Balb/c mice were prepared in a similar manner. NS-1 myeloma cells, kept in log phase in RPMI with 11% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah) for three days prior to fusion, were centrifuged at 200 g for 5 minutes, and the pellet was washed twice as described in the foregoing paragraph.

One x 10^8 spleen cells were combined with 2.0 x 10^7 NS-1 cells, centrifuged and the supernatant was aspirated. The cell pellet was dislodged by tapping the tube and 1 ml of 37°C PEG 1500 (50% in 75mM Hepes, pH 8.0) (Boehringer Mannheim) was added with stirring over the course of 1 minute, followed by adding 7 ml of serum free RPMI over 7 minutes. An additional 8 ml RPMI was added and the cells were centrifuged at 200 g for 10 minutes. After discarding the supernatant, the pellet was resuspended in 200 ml RPMI containing 15% FBS, 100 μM sodium hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine (HAT) (Gibco), 25 units/ml IL-6 (Boehringer Mannheim) and 1.5 x 10^6 thymocytes/ml and plated into 10 Corning flat bottom 96 well tissue culture plates (Corning, Corning New York).

On days 2, 4, and 6, after the fusion, 100 μl of medium was removed from the wells of the fusion plates and replaced with fresh medium. On day 8, the fusion was screened by ELISA, testing for the presence of mouse IgG binding to recombinant PAF-AH. Immulon 4 plates (Dynatech, Cambridge, MA) were coated for 2 hours at 37°C with 100 ng/well recombinant PAF-AH diluted in 25mM TRIS, pH 7.5. The coating solution was aspirated and 200ul/well of blocking solution [0.5% fish skin gelatin (Sigma) diluted in CMF-PBS] was added and incubated for 30 minutes at 37°C. Plates were washed three times with PBS with 0.05% Tween 20 (PBST) and 50 μl culture supernatant was added. After incubation at 37°C for 30 minutes, and washing as above, 50 μl of horseradish peroxidase conjugated goat anti-mouse IgG(fc) (Jackson ImmunoResearch, West Grove, Pennsylvania) diluted 1:3500 in PBST was added. Plates were incubated as above, washed four times with PBST and 100 μL substrate, consisting of 1 mg/ml o-phenylene diamine (Sigma) and 0.1 μl/ml 30% H_2O_2 in 100 mM Citrate, pH 4.5, was added. The color reaction was

stopped in 5 minutes with the addition of 50 μl of 15% H_2SO_4. A_{490} was read onn a plate reader (Dynatech).

Selected fusion wells were cloned twice by dilution into 96 well plates and visually scoring the number of colonies/well after 5 days. Hybridomas cloned were 90D1E, 90E3A, 90E6C, 90G11D (ATCC HB 11724), and 90F2D (ATCC HB 11725).

The monoclonal antibodies produced by hybridomas were isotyped using the Isostrip system (Boehringer Mannheim, Indianapolis, IN). Results showed that the monoclonal antibodies produced by hybridomas from fusion 90 were all IgG_1.

All of the monoclonal antibodies produced by hybridomas from fusion 90 functioned well in ELISA assays but were unable to bind PAF-AH on Western blots. To generate antibodies that could recognize PAF-AH by Western, mouse #1958 was immunized with recombinant enzyme. Hybridomas were generated as described for fusion 90 but were screened by Western blotting rather than ELISA to identify Western-competent clones.

For Western analyses, recombinant PAF-AH was mixed with an equal volume of sample buffer containing 125mM Tris, pH 6.8, 4% SDS, 100mM dithiothreitol and 0.05% bromphenol blue and boiled for five minutes prior to loading onto a 12% SDS polyacrylamide gel (Novex). Following electrophoresis at 40 mAmps, proteins were electrotransferred onto a polyvinylidene fluoride membrane (Pierce) for 1 hour at 125 V in 192mM glycine, 25mM Tris base, 20% methanol, and 0.01% SDS. The membrane was incubated in 20mM Tris, 100mM NaCl (TBS) containing 5% bovine serum albumin (BSA, Sigma) overnight at 4°C. The blot was incubated 1 hour at room temperature with rabbit polyclonal antisera diluted 1/8000 in TBS containing 5% BSA, and then washed with TBS and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG in TBS containing 5% BSA for 1 hour at room temperature. The blot was again washed with TBS then incubated with 0.02% 5-bromo-4-chloro-3-indolyl phosphate and 0.03% nitroblue tetrazolium in 100mM Tris-HCl, pH 9.5, 100mM NaCl, and 5mM $MgCl_2$. The reaction was stopped with repeated water rinses.

Selected fusion wells, the supernatants of which were positive in Western analyses, were processed as described above. Hybridoma 143A reacted with PAF-AH in Western blots and was cloned (ATCC HB 11900).

Polyclonal antisera specific for human plasma PAF-AH was raised in rabbits by three monthly immunizations with 100 μg of purified recombinant enzyme in Fruend's adjuvant.

Example 14

Experimental studies were performed to evaluate the *in vivo* therapeutic effects of recombinant PAF-AH of the invention on acute inflammation using a rat foot edema model [Henriques *et al.*, *Br. J. Pharmacol.*, *106*: 579-582 (1992)]. The results of these studies demonstrated that PAF-AH blocks PAF-induced edema. Parallel studies were done to compare the effectiveness of PAF-AH with two commercially available PAF antagonists.

A. Preparation of PAF-AH

E. coli transformed with the PAF-AH expression vector puc trp AH were lysed in a microfluidizer, solids were centrifuged out and the cell supernatants were loaded onto a S-Sepharose column (Pharmacia). The column was washed extensively with buffer consisting of 50mM NaCl, 10mM CHAPS, 25mM MES and 1mM EDTA, pH 5.5. PAF-AH was eluted by increasing the NaCl concentration of the buffer to 1M. Affinity chromatography using a Blue Sepharose column (Pharmacia) was then used as an additional purification step. Prior to loading the PAF-AH preparation on the Blue Sepharose column, the sample was diluted 1:2 to reduce the NaCl concentration to 0.5M and the pH was adjusted to 7.5. After washing the Blue Sepharose column extensively with buffer consisting of 0.5M NaCl, 25mM tris, 10mM CHAPS and 1mM EDTA, pH 7.5 the PAF-AH was eluted by increasing the NaCl concentration to 3.0M.

Purity of PAF-AH isolated in this manner was generally 95% as assessed by SDS-PAGE with activity in the range of 5000-10,000 U/ml. Additional quality controls done on each PAF-AH preparation included determining endotoxin levels and hemolysis activity on freshly obtained rat erythrocytes. A buffer containing 25mM Tris, 10mM CHAPS, 0.5M NaCl, pH 7.5 functioned as storage

media of the enzyme as well as carrier for administration. Dosages used in experiments were based on enzyme activity assays conducted immediately prior to experiments.

B. Induction of Edema

Six to eight-week-old female Long Evans rats (Charles River, Wilmington, MA), weighing 180-200 grams, were used for all experiments. Prior to experimental manipulations, animals were anesthetized with a mixture of the anesthetics Ketaset (Fort Dodge Laboratories, Fort Dodge, IA), Rompun (Miles, Shawnee Mission, KS), and Ace Promazine (Aveco, Fort Dodge, IA) administered subcutaneously at approximately 2.5 mg Ketaset, 1.6 mg Rompun, 0.2 mg Ace Promazine per animal per dose. Edema was induced in the foot by administration of either PAF or zymosan as follows. PAF (Sigma #P-1402) was freshly prepared for each experiment from a 19.1mM stock solution stored in chloroform/methanol (9:1) at -20°C. Required volumes were dried down under N_2, diluted 1:1000 in a buffer containing 150mM NaCl, 10mM Tris pH 7.5, and 0.25% BSA, and sonicated for five minutes. Animals received 50 μl PAF (final dose of 0.96 nmoles) subcutaneously between the hind foot pads, and edema was assessed after 1 hour and again after 2 hours in some experiments. Zymosan A (Sigma #A-8800) was freshly prepared for each experiment as a suspension of 10 mg/ml in PBS. Animals received 50 μl of zymosan (final dose of 500 μg) subcutaneously between the hind foot pads and edema was assessed after 2 hours.

Edema was quantitated by measuring the foot volume immediately prior to administration of PAF or zymosan and at indicated time point post-challenge with PAF or zymosan. Edema is expressed as the increase in foot volume in milliliters. Volume displacement measurements were made on anesthetized animals using a plethysmometer (UGO Basile, model #7150) which measures the displaced water volume of the immersed foot. In order to insure that foot immersion was comparable from one time point to the next, the hind feet were marked in indelible ink where the hairline meets the heel. Repeated measurements of the same foot using this technique indicate the precision to be within 5%.

C. PAF-AH Administration Routes and Dosages

PAF-AH was injected locally between the foot pads, or systematically by IV injection in the tail vein. For local administration rats received 100 μl PAF-AH (4000-6000 U/ml) delivered subcutaneously between the right hind foot pads. Left feet served as controls by administration of 100 μl carrier (buffered salt solution). For systemic administration of PAF-AH, rats received the indicated units of PAF-AH in 300 μl of carrier administered IV in the tail vein. Controls received the appropriate volume of carrier IV in the tail vein.

D. Local Administration of PAF-AH

Rats (N=4) were injected with 100 μl of PAF-AH (4000-6000 U/ml) subcutaneously between the right foot pads. Left feet were injected with 100 μl carrier (buffered salt solution). Four other rats were injected only with carrier. All rats were immediately challenged with PAF via subcutaneous foot injection and foot volumes assessed 1 hour post-challenge. FIGURE 4, wherein edema is expressed as average increase in foot volume (ml) ± SEM for each treatment group, illustrates that PAF-induced foot edema is blocked by local administration of PAF-AH. The group which received local PAF-AH treatment prior to PAF challenge showed reduced inflammation compared to the control injected group. An increase in foot volume of 0.08 ml ± 0.08 (SEM) was seen in the PAF-AH group as compared to 0.63 ± 0.14 (SEM) for the carrier treated controls. The increase in foot volume was a direct result of PAF injection as animals injected in the foot only with carrier did not exhibit an increase in foot volume.

E. Intravenous Administration of PAF-AH

Rats (N=4 per group) were pretreated IV with either PAF-AH (2000 U in 300 μl carrier) or carrier alone, 15 minutes prior to PAF challenge. Edema was assessed 1 and 2 hours after PAF challenge. FIGURE 5, wherein edema is expressed as average increase in volume (ml) ± SEM for each treatment group, illustrates that IV administration of PAF-AH blocked PAF induced foot edema at one and two hours post challenge. The group which received 2000 U of PAF-AH given by the IV route showed a reduction in inflammation over the two hour time course. Mean volume increase for the PAF-AH treated group at two hours was 0.10 ml ± 0.08 (SEM), versus 0.56 ml ± 0.11 for carrier treated controls.

F. Comparison of PAF-AH Protection in Edema Induced by PAF or Zymosan

Rats (N=4 per group) were pretreated IV with either PAF-AH (2000 U in 300 μl carrier) or carrier alone. Fifteen minutes after pretreatment, groups received either PAF or zymosan A, and foot volume was assessed after 1 and 2 hours, respectively. As shown in FIGURE 6, wherein edema is expressed as average increase in volume (ml) ± SEM for each treatment group, systemic administration of PAF-AH (2000 U) was effective in reducing PAF-induced foot edema, but failed to block zymosan induced edema. A mean increase in volume of 0.08 ± 0.02 was seen in the PAF-AH treated group versus 0.49 ± 0.03 for the control group.

G. Effective Dose Titration of PAF-AH Protection

In two separate experiments, groups of rats (N=3 to 4 per group) were pretreated IV with either serial dilutions of PAF-AH or carrier control in a 300 μl volume, 15 minutes prior to PAF challenge. Both feet were challenged with PAF (as described above) and edema was assessed after 1 hour. FIGURE 7 wherein edema is expressed as average increase in volume (ml) ± SEM for each treatment group, illustrates the increase in protection from PAF-induced edema in rats injected with increasing dosages of PAF-AH. In the experiments, the ID_{50} of PAF-AH given by the IV route was found to be between 40 and 80 U per rat.

H. *In Vivo* Efficacy of PAF-AH as a Function of Time After Administration

In two separate experiments, two groups of rats (N=3 to 4 per group) were pretreated IV with either PAF-AH (2000 U in 300 μl carrier) or carrier alone. After administration, groups received PAF at time points ranging from 15 minutes to 47 hours post PAF-AH administration. Edema was then assessed 1 hour after PAF challenge. As shown in FIGURE 8, wherein edema is expressed as average increase in volume (ml) ± SEM for each treatment group, administration of 2000 U of PAF-AH protects rats from PAF induced edema for at least 24 hours.

I. Pharmacokinetics of PAF-AH

Four rats received 2000 U of PAF-AH by IV injection in a 300 μl volume. Plasma was collected at various time points and stored at 4°C and plasma concentrations of PAF-AH were determined by ELISA using a double mAb capture assay. In brief, monoclonal antibody 90G11D (Example 13) was diluted in 50mM carbonate buffer pH 9.6 at 100 ng/ml and immobilized on Immulon 4 ELISA plates

overnight at 4°C. After extensive washing with PBS containing 0.05% Tween 20, the plates were blocked for 1 hour at room temperature with 0.5% fish skin gelatin (Sigma) diluted in PBS. Serum samples diluted in PBS with 15mM CHAPS were added in duplicate to the washed ELISA plate and incubated for 1 hour at room temperature. After washing, a biotin conjugate of monoclonal antibody 90F2D (Example 13) was added to the wells at a concentration of 5 μg/ml diluted in PBS and incubated for 1 hour at room temperature. After washing, 50 μl of a 1:1000 dilution of ExtraAvidin (Sigma) was added to the wells and incubated for 1 hour at room temperature. After washing, wells were developed using OPD as a substrate and quantitated. Enzyme activity was then calculated from a standard curve. FIGURE 9, wherein data points represent means ± SEM, shows that at one hour plasma enzyme levels approached the predicted concentration based on a 5-6 ml plasma volume for 180-200 gram rats, mean = 374 U/ml ± 58.2. Beyond one hour plasma levels steadily declined, reaching a mean plasma concentration of 19.3 U/ml ± 3.4 at 24 hours, which is still considerably higher than endogenous rat PAF-AH levels which have been found to be approximately 4 U/ml by enzymatic assays.

J. Effectiveness of PAF-AH Versus PAF Antagonists

Groups of rats (N=4 per group) were pretreated with one of three potential antiinflammatories: the PAF antagonist CV3988 (Biomol #L-103) administered IP (2 mg in 200 μl EtOH), the PAF antagonist Alprazolam (Sigma #A-8800) administered IP (2 mg in 200 μl EtOH), or PAF-AH (2000 U) administered IV. Control rats were injected IV with a 300 μl volume of carrier. The PAF antagonists were administered IP because they are solubilized in ethanol. Rats injected with either CV3988 or Alprazolam were challenged with PAF 30 minutes after administration of the PAF antagonist to allow the PAF antagonist to enter circulation, while PAF-AH and carrier-treated rats were challenged 15 minutes after enzyme administration. Rats injected with PAF-AH exhibited a reduction in PAF-induced edema beyond that afforded by the established PAF antagonists CV3988 and Alprazolam. See FIGURE 10 wherein edema is expressed as average increase in volume (ml) ± SEM for each treatment group.

In summary, PAF-AH is effective in blocking edema mediated by PAF *in vivo*. Administration of PAF-AH can be either local or systemic by IV injection.

In dosing studies, IV injections in the range of 160-2000 U/rat were found to dramatically reduce PAF mediated inflammation, while the ID_{50} dosage appears to be in the range of 40-80 U/rat. Calculations based on the plasma volume for 180-200 gram rats predicts that a plasma concentration in the range of 25-40 U/ml should block PAF-elicited edema. These predictions are supported by preliminary pharmacokinetic studies. A dosage of 2000 U of PAF-AH was found to be effective in blocking PAF mediated edema for at least 24 hours. At 24 hours following administration of PAF-AH plasma concentrations of the enzyme were found to be approximately 25 U/ml. PAF-AH was found to block PAF-induced edema more effectively than the two known PAF antagonists tested.

Collectively, these results demonstrate that PAF-AH effectively blocks PAF induced inflammation and may be of therapeutic value in diseases where PAF is the primary mediator.

Example 15

Recombinant PAF-AH of the invention was tested in a second *in vivo* model, PAF-induced pleurisy. PAF has previously been shown to induce vascular leakage when introduced into the pleural space [Henriques *et al., supra*]. Female rats (Charles River, 180-200 g) were injected in the tail vein with 200 μl of 1% Evans blue dye in 0.9% with 300 μl recombinant PAF-AH (1500 μmol/ml/hour, prepared as described in Example 14) or with an equivalent volume of control buffer. Fifteen minutes later the rats received an 100 μl injection of PAF (2.0 nmol) into the pleural space. One hour following PAF challenge, rats were sacrificed and the pleural fluid was collected by rinsing the cavity with 3 ml heparinized phosphate buffered saline. The degree of vascular leak was determined by the quantity of Evans blue dye in the pleural space which was quantitated by absorbance at 620 nm. Rats pretreated with PAF-AH were found to have much less vascular leakage than control animals (representing more than an 80% reduction in inflammation).

The foregoing results support the treatment of subjects suffering from pleurisy with recombinant PAF-AH enzyme of the invention.

Example 16

Recombinant PAF-AH enzyme of the invention was also tested for efficacy in a model of antigen-induced eosinophil recruitment. The accumulation of eosinophils in the airway is a characteristic feature of late phase immune responses which occur in asthma, rhinitis and eczema. BALB/c mice (Charles River) were sensitized by two intraperitoneal injections consisting of 1 μg of ovalbumin (OVA) in 4 mg of aluminum hydroxide (Imject alum, Pierce Laboratories, Rockford, IL) given at a 2 week interval. Fourteen days following the second immunization, the sensitized mice were challenged with either aerosolized OVA or saline as a control.

Prior to challenge mice were randomly placed into four groups, with four mice/group. Mice in groups 1 and 3 were pretreated with 140 μl of control buffer consisting of 25mM tris, 0.5M NaCl, 1mM EDTA and 0.1% Tween 80 given by intravenous injection. Mice in groups 2 and 4 were pretreated with 750 units of PAF-AH (activity of 5,500 units/ml given in 140 μl of PAF-AH buffer). Thirty minutes following administration of PAF-AH or buffer, mice in groups 1 and 2 were exposed to aerosolized PBS as described below, while mice in groups 3 and 4 were exposed to aerosolized OVA. Twenty-four hours later mice were treated a second time with either 140 μl of buffer (groups 1 and 3) or 750 units of PAF-AH in 140 μl of buffer (groups 2 and 4) given by intravenous injection.

Eosinophil infiltration of the trachea was induced in the sensitized mice by exposing the animals to aerosolized OVA. Sensitized mice were placed in 50 ml conical centrifuge tubes (Corning) and forced to breath aerosolized OVA (50 mg/ml) dissolved in 0.9% saline for 20 minutes using a nebulizer (Model 646, DeVilbiss Corp., Somerset, PA). Control mice were treated in a similar manner with the exception that 0.9% saline was used in the nebulizer. Forty-eight hours following the exposure to aerosolized OVA or saline, mice were sacrificed and the tracheas were excised. Tracheas from each group were inbeded in OCT and stored at -70° until sections were cut.

To evaluate eosinophil infiltration of the trachea, tissue sections from the four groups of mice were stained with either Luna solution and hematoxylin-eosin solution or with peroxidase. Twelve 6 μm thick sections were cut fr m each group of mice and numbered accordingly. Odd numbered sections were stained with Luna

stain as follows. Sections were fixed in formal-alcohol for 5 minutes at room temperature, rinsed across three changes of tap water for 2 minutes at room temperature then rinsed in two changed of dH_2O for 1 minute at room temperature. Tissue sections were stained with Luna stain 5 minutes at room temperature (Luna stain consisting of 90 ml Weigert's Iron hematoxylin and 10 ml of 1% Biebrich Scarlet). Stained slides were dipped in 1% acid alcohol six times, rinsed in tap water for 1 minute at room temperature, dipped in 0.5% lithium carbonate solution five times and rinsed in running tap water for 2 minutes at room temperature. Slides were dehydrated across 70%-95%-100% ethanol 1 minute each, at room temperature, then cleared in two changes of xylene for 1 minute at room temperature and mounted in Cytoseal 60.

For the peroxidase stain, even numbered sections were fixed in 4°C acetone for 10 minutes and allowed to air dry. Two hundred μl of DAB solution was added to each section and allowed to sit 5 minutes at room temperature. Slides were rinsed in tap water for 5 minutes at room temperature and 2 drops of 1% osmic acid was applied to each section for 3-5 seconds. Slides were rinsed in tap water for 5 minutes at room temperature and counterstained with Mayers hematoxylin at 25°C at room temperature. Slides were then rinsed in running tap water for 5 minutes and dehydrated across 70%-95%-100% ethanol 1 minute each at room temperature. Slides were cleared through two changes of xylene for 1 minute each at room temperature and mounted in Cytoseal 60.

The number of eosinophils in the submucosal tissue of the trachea was evaluated. Trachea from mice from groups 1 and 2 were found to have very few eosinophils scattered throughout the submucosal tissue. As expected tracheas from mice in group 3, which were pretreated with buffer and exposed to nebulized OVA, were found to have large numbers of eosinophils throughout the submucosal tissue. In contrast, the tracheas from mice in group 4, which were pretreated with PAF-AH and exposed to nebulized OVA were found to have very few eosinophils in the submucosal tissue comparable to what was seen in the two control groups, groups 1 and 2.

Thus, therapeutic treatment with PAF-AH of subjects exhibiting a late phase immune response involving the accumulation of eosinophils in the airway, such as that which occurs in asthma and rhinitis is indicated.

Example 17

PAF-AH of the invention was also tested in a rat model for treatment of necrotizing enterocolitis (NEC), an acute hemorrhagic necrosis of the bowel which occurs in low birth weight infants and causes a significant morbidity and mortality. Previous experiments have demonstrated that treatment with glucocorticoids decreases the incidence of NEC in animals and in premature infants, and the activity of glucocorticoids has been suggested to occur via an increase in the activity of plasma PAF-AH.

A. Prevention of NEC

Recombinant PAF-AH (25,500 units in 0.3 ml, groups 2 and 4) or vehicle/buffer alone (25mM tris, 0.5M NaCl, 1mM EDTA and 0.1% Tween 80) (groups 1 and 3) was administered into the tail veins of female Wistar rats (n=3) weighing 180-220 grams. Either BSA (0.25%)- saline (groups 1 and 2) or PAF (0.2 μg/100 gm) suspended in BSA saline (groups 3 and 4) was injected into the abdominal aorta at the level of the superior mesenteric artery 15 minutes after PAF-AH or vehicle injection as previously described by Furukawa, *et al.* [*J.Pediatr.Res.* *34*:237-241 (1993)]. The small intestines were removed after 2 hours from the ligament of Trietz to the cecum, thoroughly washed with cold saline and examined grossly. Samples were obtained from microscopic examination from the upper, middle and lower portions of the small intestine. The tissues were fixed in buffered formalin and the sample processed for microscopic examination by staining with hematoxylin and eosin. The experiment was repeated three times.

Gross findings indicated a normal appearing bowel in groups treated with the vehicle of BSA saline. Similarly, PAF-AH injected in the absence of PAF had no effect on the gross findings. In contrast, the injection of PAF into the descending aorta resulted in rapid, severe discoloration and hemorrhage of the serosal surface of the bowel. A similar hemorrhage was noted when a section of the small bowel was examined on the mucosal side and the intestine appeared to be quite

necrotic. When PAF-AH was injected via the tail vein 15 minutes prior to the administration of PAF into the aorta the bowel appeared to be normal.

Upon microscopic examination, the intestine obtained from groups 1, 2 and 4 demonstrated a normal villous architecture and a normal population of cells within the lamina propria. In contrast, the group treated with PAF alone showed a full thickness necrosis and hemorrhage throughout the entire mucosa.

The plasma PAF-AH activities were also determined in the rats utilized in the experiment described above. PAF-AH activity was determined as follows. Prior to the tail vein injection, blood samples were obtained. Subsequently blood samples were obtained from the vena cava just prior to the injection of PAF and at the time of sacrifice. Approximately 50 μl of blood was collected in heparinized capillaries. The plasma was obtained following centrifugation (980 x *g* for 5 minutes). The enzyme was assayed as previously described by Yasuda and Johnston, *Endocrinology, 130*:708-716 (1992).

The mean plasma PAF-AH activity of all rats prior to injection was found to be 75.5 $\pm$ 2.5 units (1 unit equals 1 nmoles x min^{-1} x ml^{-1} plasma). The mean plasma PAF-AH activities 15 minutes following the injection of the vehicle were 75.2 $\pm$ 2.6 units for group 1 and 76.7 $\pm$ 3.5 units for group 3. After 15 minutes, the plasma PAF-AH activity of the animals injected with 25,500 units recombinant PAF-AH was 2249 $\pm$ 341 units for group 2 and 2494 $\pm$ 623 units for group 4. The activity of groups 2 and 4 remained elevated (1855 $\pm$ 257 units) until the time of sacrifice (2 1/4 hours after PAF-AH injection) (Group 2 = 1771 $\pm$ 308; Group 4 = 1939 $\pm$ 478). These results indicate that plasma PAF-AH activity of the rats which were injected with the vehicle alone (groups 1 and 3) did not change during the course of the experiment. All the animals receiving the PAF injection alone developed NEC while all rats that were injected with PAF-AH followed by PAF injection were completely protected.

B. Dose-Dependency of Prevention of NEC

In order to determine if the protection against NEC in rats was dose dependent, animals were treated with increasing doses of PAF-AH 15 minutes prior to PAF administration. Initially, PAF-AH, ranging from 25.5 to 25,500 units were administered into the tail vein of rats. PAF (0.4 μg in 0.2 ml of BSA-saline) was

subsequently injected into the abdominal aorta 15 minutes after the administration of PAF-AH. The small intestine was removed and examined for NEC development 2 hours after PAF administration. Plasma PAF-AH activity was determined prior to the exogenous administration of the enzyme, and 15 minutes and 2 1/4 hours after PAF-AH administration. The results are the mean of 2-5 animals in each group.

Gross findings indicated that all rats receiving less than 2,000 units of the enzyme developed NEC. Plasma PAF-AH activity in animals receiving the lowest protective amount of enzyme (2040 units) was 363 units per ml of plasma after 15 minutes, representing a five-fold increase over basal levels. When PAF-AH was administered at less than 1,020 total units, resultant plasma enzyme activity averaged approximately 160 or less, and all animals developed NEC.

C. Duration of Protection Against NEC

In order to determine the length of time exogenous PAF-AH afforded protection against development of NEC, rats were injected once with a fixed amount of the enzyme via the tail vein and subsequently challenged with PAF at various time points. PAF-AH (8,500 units in 0.3 ml) or vehicle alone was administered into the tail vein of rats, and PAF (0.36 μg in 0.2 ml of BSA-saline) was injected into the abdominal aorta at the various times after the enzyme administration. The small intestines were removed 2 hours after the PAF injection for gross and histological examinations in order to evaluate for NEC development. Plasma PAF-AH activities were determined at various times after enzyme administration and two hours after PAF administration. The mean value $\pm$ standard error for enzyme activity was determined for each group.

Results indicated that none of the rats developed NEC within the first eight hours after injection of PAF-AH, however 100% of the animals challenged with PAF at 24 and 48 hours following injection of the enzyme developed NEC.

D. Reversal of NEC

In order to determine if administration of PAF-AH was capable of reversing development of NEC induced by PAF injection, 25,500 units of enzyme was administered via injection into the vena cava two minutes following PAF administration (0.4 μg). None of the animals developed NEC. However, when PAF-

AH was administered via this route 15 minutes after the PAF injection. all animals developed NEC, consistent with the rapid time course of NEC development as induced by the administration of PAF previously reported Furukawa *et al.* [*supra*].

The sum of these observations indicate that a relatively small (five-fold) increase in the plasma PAF-AH activity is capable of preventing NEC. These observations combined with previous reports that plasma PAF-AH activity in fetal rabbits [Maki, *et al.*, *Proc.Natl.Acad.Sci.(USA)* *85*:728-732 (1988)] and premature infants [Caplan, *et al.*, *J.Pediatr.* *116*:908-964 (1990)] has been demonstrated to be relatively low suggests that prophylactic administration of human recombinant PAF-AH to low birth weight infants may be useful in treatment of NEC.

Example 18

The efficacy of PAF-AH in a guinea pig model of acute respiratory distress syndrome (ARDS) was examined.

Platelet-activating factor (PAF) injected intravenously into guinea pigs produces a profound lung inflammation reminiscent of early ARDS in humans. Within minutes after intravenous administration of PAF, the lung parenchyma becomes congested with constricted bronchi and bronchioles [Lellouch-Tubiana *et al.*, *supra*. Platelets and polymorphonuclear neutrophils begin to marginate and cellular aggregates are easily identified along arterioles of the lung [Lellouch-Tubiana, *Br. J. Exp Path.*, *66*:345-355 (1985)]. PAF infusion also damages bronchial epithelial cells which dissociate from the airway walls and accumulate in the airway lumens. This damage to airway epithelial cells is consistent with hyaline membrane formation that occurs in humans during the development of ARDS. Margination of the neutrophils and platelets is quickly followed by diapedesis of these cells into the alveolar septa and alveolar spaces of the lung. Cellular infiltrates elicited by PAF are accompanied by significant vascular leakage resulting in airway edema [Kirsch, *Exp. Lung Res.*, *18*:447-459 (1992)]. Evidence of edema is further supported by *in vitro* studies where PAF induces a dose-dependent (10-1000 ng/ml) extravasation of ^{125}I labeled fibrinogen in perfused guinea pig lungs [Basran, *Br. J. Pharmacol.*, *77*:437 (1982)].

Based on the above observations, an ARDS model in guinea pigs was developed. A cannula is placed into the jugular vein of anaesthetized male Hartly guinea pigs (approximately 350-400 grams) and PAF diluted in a 500 μl volume of phosphate buffered saline with 0.25% bovine serum albumin as a carrier (PBS-BSA) is infused over a 15 minute period of time at a total dosage ranging from 100-400 ng/kg. At various intervals following PAF infusion, animals are sacrificed and lung tissue is collected. In guinea pigs infused with PAF, dose dependent lung damage and inflammation is clearly evident by 15 minutes and continues to be present at 60 minutes. Neutrophils and red blood cells are present in the alveolar spaces of PAF treated guinea pigs but absent in control or sham infused animals. Evidence of epithelial cell damage is also evident and reminiscent of hyaline membrane formation in human ARDS patients. Protein determinations done on bronchoalveolar lavage (BAL) samples taken from guinea pigs infused with PAF shows a dramatic accumulation of protein in the inflamed lung, clear evidence of vascular leakage.

PAF-AH was found to completely protect against PAF mediated lung injury in the guinea pig model of ARDS. Groups of guinea pigs were pretreated with either PAF-AH (2000 units in 500 μl) or 500 μl of the PAF-AH buffer only. Fifteen minutes later these guinea pigs were infused with 400 ng/kg PAF in a 500 μl volume, infused over a 15 minute period. In addition, a sham group of guinea pigs was infused with 500 μl of PBS-BSA. At the completion of the PAF infusion the animals were sacrificed and BAL fluid was collected by lavaging the lungs 2X with 10ml of saline containing 2 μ/ml heparin to prevent clotting. To determine protein concentration in the BAL, samples were diluted 1:10 in saline and the OD 280 was determined. BAL fluid from sham guinea pigs was found to have a protein concentration of 2.10 $\pm$ 1.3 mg/ml. In sharp contrast, BAL fluid from animals infused with PAF was found to have a protein concentration of 12.55 $\pm$ 1.65 mg/ml. In guinea pigs pretreated with PAF-AH, BAL fluid was found to have a protein concentration of 1.13 $\pm$ 0.25 mg/ml which is comparable to the sham controls and demonstrates that PAF-AH completely blocks lung edema in response to PAF.

Example 19

Nearly four percent of the Japanese population has low or undetectable levels of PAF-AH activity in their plasma. This deficiency has been correlated with severe respiratory symptoms in asthmatic children [Miwa *et al.*, *J. Clin. Invest*,. 82: 1983-1991 (1988)] who appear to have inherited the deficiency in an autosomal recessive manner.

To determine if the deficiency arises from an inactive but present enzyme or from an inability to synthesize PAF-AH, plasma from multiple patients deficient in PAF-AH activity was assayed both for PAF-AH activity (by the method described in Example 10 for transfectants) and for the presence of PAF-AH using the monoclonal antibodies 90G11D and 90F2D (Example 13) in a sandwich ELISA as follows. Immulon 4 flat bottom plates (Dynatech, Chantilly, VA) were coated with 100 ng/well of monoclonal antibody 90G11D and stored overnight. The plates were blocked for 1 hour at room temperature with 0.5% fish skin gelatin (Sigma) diluted in CMF-PBS and then washed three times. Patient plasma was diluted in PBS containing 15mM CHAPS and added to each well of the plates (50 μl/well). The plates were incubated for 1 hour at room temperature and washed four times. Fifty μl of 5 μg/ml monoclonal antibody 90F2D, which was biotinylated by standard methods and diluted in PBST, was added to each well, and the plates were incubated for 1 hour at room temperature and then washed three times. Fifty μl of ExtraAvidin (Sigma) diluted 1/1000 in CMF-PBST was subsequently added to each well and plates were incubated for 1 hour at room temperature before development.

A direct correlation between PAF-AH activity and enzyme levels was observed. An absence of activity in a patient's serum was reflected by an absence of detectable enzyme. Similarly, plasma samples with half the normal activity contained half the normal levels of PAF-AH. These observations suggested that the deficiency of PAF-AH activity was due to an inability to synthesize the enzyme or due to an inactive enzyme which the monoclonal antibodies did not recognize.

Further experiments revealed that the deficiency was due to a genetic lesion in the human plasma PAF-AH gene. Genomic DNA from PAF-AH deficient individuals was isolated and used as template for PCR reactions with PAF-AH gene specific primers. Each of the coding sequence exons were initially amplified and

sequenced from one individual. A single nucleotide change within exon 9 was observed (a G to T at position 996 of SEQ ID NO: 7). The nucleotide change results in an amino acid substitution of a phenylalanine for a valine at position 279 of the PAF-AH sequence (V279F). Exon 9 was amplified from genomic DNA from an additional eleven PAF-AH deficient individuals who were found to have the same point mutation.

To test whether this mutation crippled the enzyme, an *E. coli* expression construct containing the mutation was generated by methods similar to that described in Example 10. When introduced into *E. coli*, the expression construct generated no PAF-AH activity while a control construct lacking the mutation was fully active. This amino acid substitution presumably results in a structural modification which causes the observed deficiency of activity and lack of immunoreactivity with the PAF-AH antibodies of the invention.

PAF-AH specific antibodies of the invention may thus be used in diagnostic methods to detect abnormal levels of PAF-AH in serum (normal levels are about 1 to 5 U/ml) and to follow the progression of treatment of pathological conditions with PAF-AH. Moreover, identification of a genetic lesion in the PAF-AH gene allows for genetic screening for the PAF-AH deficiency exhibited by the Japanese patients. The mutation causes the gain of a restriction endonuclease site (Mae II) and thus allows for the simple method of Restriction Fragment Length Polymorphism (RFLP) analysis to differentiate between active and mutant alleles. See Lewin, pp. 136-141 in *Genes V*, Oxford University Press, New York, New York (1994).

Screening of genomic DNA from twelve PAF-AH deficient patients was carried out by digestion of the DNA with MaeII, Southern blotting, and hybridization with an exon 9 probe (nucleotides 1-396 of SEQ ID NO: 17). All patients were found to have RFLPs consistent with the mutant allele.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those skilled in the art. Accordingly, only such limitations as appear in the appended claims should be placed on the invention.